ABSTRACT

Title of Document: **DECONSTRUCTING THE POLYCLONAL ANTIBODY RESPONSE TO DENGUE VIRUS**

Laura Anne VanBlargan, Doctor of Philosophy, 2015

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Dengue viruses are mosquito-borne flaviviruses that circulate in nature as four related serotypes (DENV1-4). These emerging pathogens are responsible for an estimated 390 million human infections each year. The outcome of human DENV infection ranges from clinically inapparent disease (~75% of infections), to a self-limiting febrile illness, to severe disease characterized by hemorrhage and shock. Severe clinical manifestations of disease are predominantly associated with secondary infections by a heterotypic DENV serotype. The increased risk of severe disease in DENV-sensitized populations significantly complicates vaccine development, as a vaccine should confer protection against all four DENV serotypes. As the development of a neutralizing antibody response is a correlate of protection for successful vaccines for several other flaviviruses, eliciting a protective tetravalent

neutralizing antibody response is a major goal of ongoing DENV vaccine development efforts. Understanding the neutralizing antibody response to infection and vaccination is an important step toward the development and evaluation of safe DENV vaccines. While considerable insights have been gained from studies of monoclonal antibodies, the individual contributions and dynamics of the repertoire of circulating antibody specificities elicited by infection and vaccination are poorly understood on a functional and molecular level. We studied human polyclonal antibody responses elicited by monovalent DENV1 vaccination and sought to identify epitopes recognized by serotype-specific neutralizing antibodies. DENV1 structural gene variants were produced and screened for reduced sensitivity to neutralization by DENV1 sera but unaltered sensitivity to control antibodies. We identified amino acid residues that contribute significantly to type-specific recognition by polyclonal DENV1 immune sera. These findings provide an important step towards deconvoluting the functional complexity of DENV serology following vaccination.

DECONSTRUCTING THE POLYCLONAL ANTIBODY RESPONSE TO DENGUE VIRUS

By

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List of Abbreviations

Ab	antibody
ADCC	antibody-dependent cellular cytotoxicity
ADE	antibody-dependent enhancement
ANOVA	analysis of variance
BCR	B cell receptor
bnAb	broadly-neutralizing antibody
C	capsid protein
CDR	complementarity determining region
CprME	structural genes C, prM, and E
CR	cross-reactive
DENV	dengue virus
DENVrep	dengue virus replicon
DF	dengue fever
DHF	dengue hemorrhagic fever
DI, DII, DIII	domain I, II, and III
DSS	dengue shock syndrome
E	envelope protein
EC50	half maximal effective concentration
ER	endoplasmic reticulum
FcγR	Fc-gamma receptors
FDC	follicular dendritic cells
FL	fusion loop
GC	germinal center
HCV	hepatitis C virus
HIV	human immunodeficiency virus
Ig	immunoglobulin
JEV	Japanese encephalitis virus
LLPC	long-lived plasma cells
M	membrane protein
mAb	monoclonal antibody
MBC	memory B cell
MPER	membrane-proximal external region
nAb	neutralizing antibody
NGC	New Guinea C
NS	non-structural protein
NS2B/3	non-structural protein 2B + 3
NT50	half maximal neutralization titer
ORF	open reading frame
PC	plasma cell

PCR	polymerase chain reaction
PDB	protein database
prM	pre-membrane protein
PRNT	plaque-reduction neutralization test
qRT-PCR	quantitative reverse transcription
RBD	receptor binding domain
RVP	reporter virus particle
SVP	subviral particle
TBEV	tick-borne encephalitis virus
TFH cell	follicular helper T cell
TS	type-specific
V	variable
WNV	West Nile virus
WNVrep	West Nile virus replicon
WP	Western Pacific-74
WT	wild type
YFV	yellow fever virus

Chapter 1: Introduction

Dengue virus (DENV) is a mosquito-transmitted flavivirus responsible for an estimated 390 million human infections each year (1). Four related serotypes (DENV1-4) circulate in virtually all tropical and sub-tropical regions of the world (2). While DENV infection is often subclinical, infected individuals may develop dengue fever (DF), which has symptoms of self-limiting febrile illness, myalgia, rash, and retro-orbital pain (3), or more severe clinical illness (dengue shock syndrome/dengue hemorrhagic fever) involving capillary leakage, thrombocytopenia, and hemorrhage. Severe disease is typically associated with secondary infections by a heterologous DENV serotype (4-7). The incidence of severe DENV disease is rising globally due to increasing co-circulation of multiple DENV serotypes in endemic areas (2, 8). Currently, there are no specific treatments or approved vaccines for DENV infection.

DENV infection elicits the production of long-term serum neutralizing antibodies (9). The neutralizing antibody response elicited following infection likely contributes to the long-lived protection against reinfection that is observed (10). However, immunity following primary infection is typically serotype-specific, and thus DENV-experienced individuals may be susceptible to secondary infection by a heterologous serotype (11). A portion of the immune response after primary infection is cross-reactive against heterologous serotypes; however, this cross-reactive response is poorly protective, and may in fact contribute to the pathogenesis of severe disease during secondary heterotypic

infections (11, 12). Therefore, a DENV vaccine should provide protection against all four serotypes to avoid the incomplete immunity that serves as a risk factor for severe disease. Because antibodies against DENV have the potential to mediate protection as well as pathogenesis of severe disease, characterizing the polyclonal antibody response to DENV infection and vaccination is an important step toward the development and evaluation of safe vaccines against DENV.

The principle goal of this dissertation was to identify epitopes involved in the type-specific neutralizing antibody response elicited by DENV1 vaccination in humans. The Laboratory of Infectious Diseases of the National Institutes of Health (NIH) is developing a live-attenuated, tetravalent dengue vaccine, and has tested several vaccine candidates and formulations in clinical studies (13). Using post-vaccination sera obtained from clinical trials of NIH DENV1 and DENV2 monovalent vaccine formulations, we developed methods to deconstruct the functional components of a polyclonal antibody response. We used a mutagenesis strategy to characterize variants that had a reduced sensitivity to neutralization by sera from vaccine recipients. Reporter virus particle (RVP) technology was employed for this study, as it streamlines the process of constructing and characterizing a large panel of structural gene plasmids. Additionally, RVPs are not passaged in cell culture, and thus the genetic stability of a particular mutation does not limit its utility in neutralization studies.

In order to study the relevant strains of DENV present in the NIH vaccine candidate, RVPs containing the structural genes of these strains had to be generated. The attenuated

DENV1 strain of the tetravalent formulation is derived from the Western Pacific (WP) strain (14), and the DENV2 component in this vaccine is derived from the New Guinea C (NGC) strain (15). However, initial experiments demonstrated that it was not possible to produce RVPs for all DENV strains in our existing method. As outlined in chapter 3, in order to study the strains of DENV present in the NIAID vaccine candidate we had to decipher why RVPs could not be generated from the required DENV strain, repair the cause of the incompatibility with RVP technology, and establish a method for making RVPs of the vaccine strains.

Following successful production of DENV RVPs corresponding to the NIH vaccine strains, we constructed a large library of DENV1 structural gene variants, which is described in Chapter 4. Using sera obtained from volunteers in a clinical trial of the NIH DENV1 monovalent vaccine, we screened the DENV1 variants for reduced sensitivity to neutralization. Using this strategy, residues on DENV1 that are important for recognition by type-specific neutralizing antibodies in polyclonal sera were identified. Variants were further characterized by control antibodies to assess whether mutations altered virion structure in a manner that would confound our interpretation of the results from the polyclonal sera study.

In chapter 5, we investigated polyclonal antibody recognition of heterologous DENV1 strains by sera from recipients of the DENV1 monovalent vaccine. Since a DENV vaccine should induce immunity against the variety of strains in each serotype that circulate in nature, it is important to characterize the potency of the antibody response to

vaccination against diverse DENV1 strains. We observed that antibodies elicited against DENV1 strain WP were capable of neutralizing heterologous strains, though often with reduced capacity relative to the strain used in immunization. Studying diverse DENV1 strains also allowed us to characterize the effect of strain-dependent differences in virion structural dynamics on the neutralizing potency of polyclonal sera. Altogether, the findings outlined in this dissertation provide insight into the neutralizing antibody response to DENV1 in the context of human polyclonal sera.

Chapter 2: Literature Review[1]

2.1 Flaviviruses

2.1.1 Classification

The genus *Flavivirus*, of the family *Flaviviridae*, is a diverse group of positive-stranded RNA viruses transmitted to vertebrate hosts principally by mosquito or tick vectors. This genus of ~70 viral species includes several viruses of considerable clinical importance, including dengue virus (DENV), yellow fever virus (YFV), West Nile virus (WNV), Japanese encephalitis virus (JEV), and tick-borne encephalitis virus (TBEV). An estimated 390 million humans are infected each year with DENV alone (1). Flaviviruses were initially classified using serological studies, which were subsequently confirmed and extended via phylogenetic analyses (16, 17). A viral species within the flavivirus genus is defined as a group of viruses sharing over 84% nucleotide sequence identity, while viruses in a clade share at least 69% nucleotide identity (17). For example, the four serotypes of dengue virus (DENV1-4) constitute a clade, with each serotype designated as an individual species within the DENV clade (**Figure 2.1**).

[1] Adapted in part from: **VanBlargan LA, Goo L, Pierson TC.** Deconstructing the Antiviral Neutralizing Antibody Response: Implications for Vaccine Development and Immunity. Manuscript submitted for publication.
[2] Adapted from reference 226. **VanBlargan LA, Davis KA, Dowd KA, Akey DL, Smith JL, Pierson TC.**

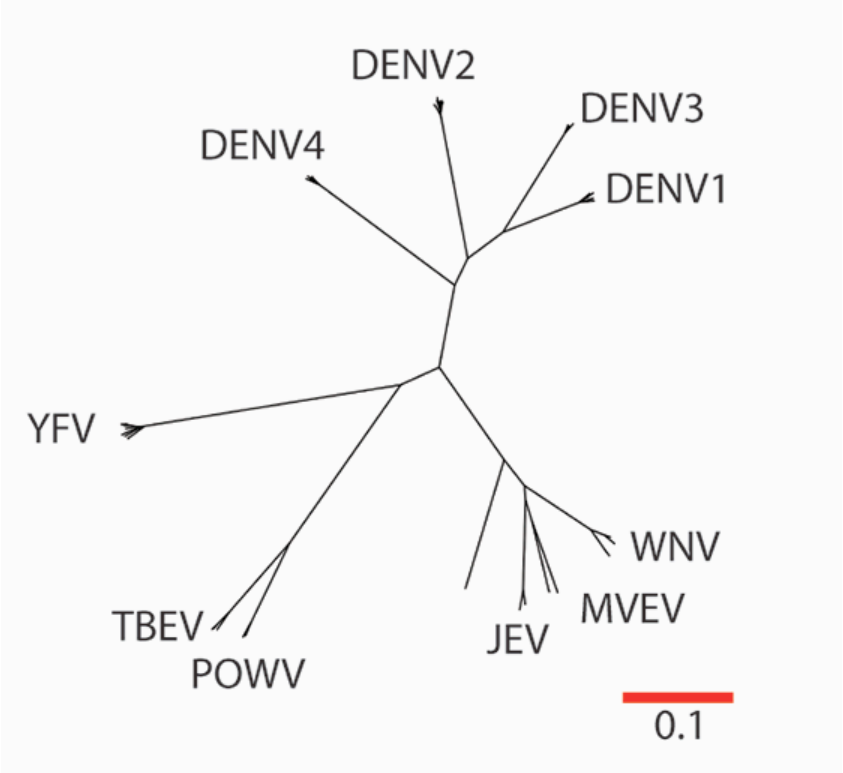


Figure 2.1 Diversity of the surface glycoproteins of flaviviruses. Dendrogram depicting the relatedness of selected flavivirus E proteins (scale bar represents 0.1 amino acid substitutions per site). JEV; Japanese encephalitis virus; MVEV, Murray Valley encephalitis virus; WNV, West Nile virus; SLEV, Saint Louis encephalitis virus; TBEV, tick-borne encephalitis virus; POWV, Powassan virus; YFV, yellow fever virus; DENV, dengue virus.

2.1.2 Viral genome organization and replication

The flavivirus genome is a single-stranded, positive-sense RNA of ~11 kb in length. The genome encodes a single open reading frame (ORF) over 10 kb in length that is flanked by 5' and 3' untranslated regions. The ORF is translated into a large polyprotein that translocates into the membrane of the endoplasmic reticulum (ER) (18). The polyprotein is co- and post-translationally cleaved by host and viral proteases into ten proteins, including the structural proteins capsid (C), pre-membrane (prM), and envelope (E) that are packaged into virions, as well as non-structural proteins NS1, NS2A, NS2B, NS3, NS4A, NS4B, and NS5 (**Figure 2.2**). Viral genome replication occurs in the perinuclear region in association with membranes derived from the ER that have undergone virus-induced ultra-structural reorganization (19). The viral non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) are present in viral replication complexes, along with viral genomic RNA, and likely host factors. Viral genome replication and genome packaging are tightly coupled both functionally and spatially (reviewed by (20)). Active genome replication has been shown to be a prerequisite for genome-packaging, as replication-deficient genomes result in no virus particle production (21). Virus assembly occurs at the ER membrane in sites that are distinct from, but in close proximity to sites of genome replication, possibly to ensure efficient shuttling of viral genomes (19).

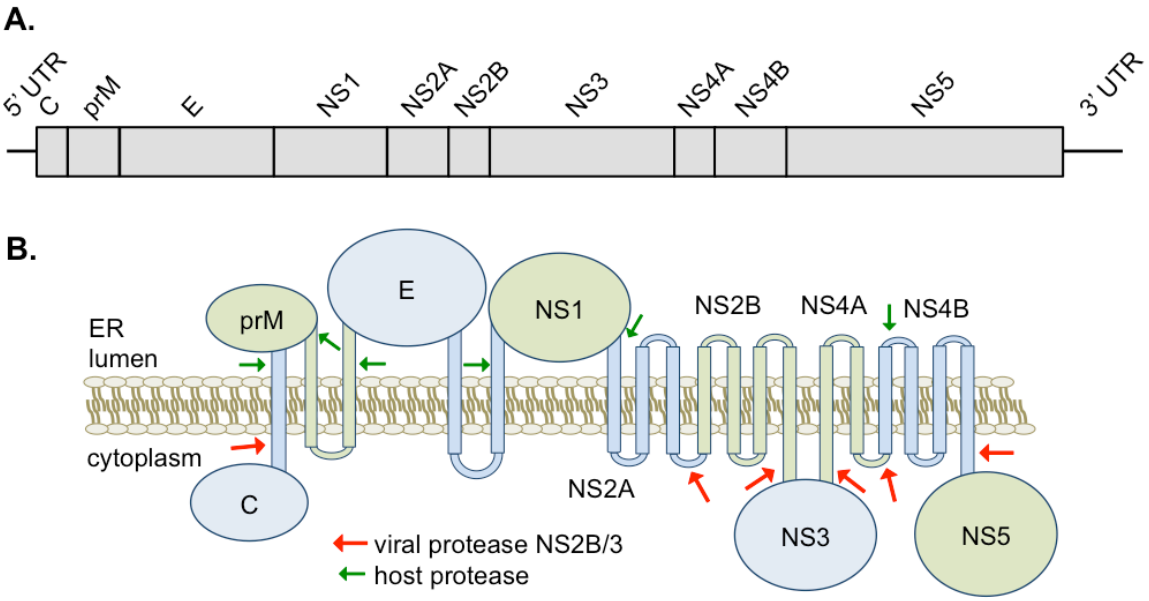


Figure 2.2 Organization of the flavivirus genome. (**A**) The ~11 kb genome of flaviviruses is organized as a single strand of positive-sense RNA that serves as the viral mRNA. The genome has 5' and 3' untranslated regions (UTR) that flank the single ORF. The ORF contains 10 genes, including the structural genes capsid (C), pre-membrane (prM), and envelope (E), as well as seven non-structural (NS) genes. (**B**) The ORF is translated into a polyprotein that spans membranes derived from the ER. Signal sequences direct translocation of glycoproteins prM, E, and NS1 into the ER lumen. The polyprotein undergoes co- and post-translational processing by host and viral proteases to yield individual viral proteins.

2.1.3 Virus assembly, budding, and maturation

Assembly of new virions is directed by the prM and E proteins. As nascent virions bud into the ER lumen, they acquire a prM- and E-studded lipid envelope that surrounds a nucleocapsid composed of C proteins in association with a viral genome (22-24). On newly formed virions, one role of prM is to prevent low pH-triggered conformational changes in the E proteins that drive fusion of viral and cellular membranes (25). These newly formed virions are immature and non-infectious, and must undergo a maturation process as they transit the secretory pathway to become mature, infectious virions. Virion maturation takes place as immature virions traffic through the secretory pathway, where cleavage of the prM protein occurs by cellular furin-like proteases (26-28). This cleavage results in a ~75 amino acid M peptide that remains associated with the mature virion, and a ~90 amino acid, soluble "pr" portion that disassociates from virus particles upon release from cells (29). The function of M on the mature, infectious virion is unknown.

Critically, the flavivirus maturation process is not efficient, resulting in the production of a heterogeneous population of virions. Thus, in addition to infectious, fully mature virions (no prM) and non-infectious, immature virions (180 uncleaved prM molecules), cells produce partially mature viruses that retain structural features of both mature and immature virus particles (30). Partially mature virions can be infectious, though the extent of prM cleavage required for infectivity is not known (reviewed by (30)). The efficiency of virion maturation has the potential to impact virus binding, environmental conditions required to trigger membrane fusion, cellular tropism, and sensitivity to antibody-mediated neutralization (31-35).

2.1.4 Flavivirus structure

Flavivirus virions are small, spherical, enveloped particles roughly 50 nm in diameter that are composed of three structural proteins: capsid (C), pre-membrane (prM), and envelope (E). The virion maturation process results not only in the loss of the pr peptide, but also in a structural reorganization of the E protein. Therefore, immature and mature virions have distinct structural organizations. On immature virions, the prM and E glycoproteins are incorporated as 60 icosahedrally-arranged heterotrimeric spikes of three prM-E dimers (29, 36-38). On mature virions, E proteins exist as a dense herringbone arrangement of 90 antiparallel E protein homodimers in T=3 pseudo-icosahedral symmetry (**Figure 2.3A and 2.3B**) (39-41). In this configuration, E proteins lie flat against the surface of the viral membrane, in contrast to many other viruses whose envelope proteins exist as spikes that project away from the virion surface.

The E protein is the main target of neutralizing antibodies (nAbs) (42). This elongated protein is composed of three distinct domains (DI-DIII) connected to the viral membrane by a helical stem and two transmembrane domains (**Figure 2.3A**) (43). A highly conserved, hydrophobic fusion loop composed of 13 amino acids is located at the distal end of DII (DII-FL). On mature virus particles, the fusion loop is buried in a fold composed of DI and DIII of the opposing E protein in the dimer (44). E proteins may contain up to two N-linked glycosylation sites (either on DI or DI and DII) (**Figure 2.3C**); some strains of WNV are non-glycosylated (45, 46). Neutralizing antibodies have been mapped to all three E protein domains, and in many instances, bind epitopes composed of residues from multiple domains (47-58). Antibodies that recognize prM

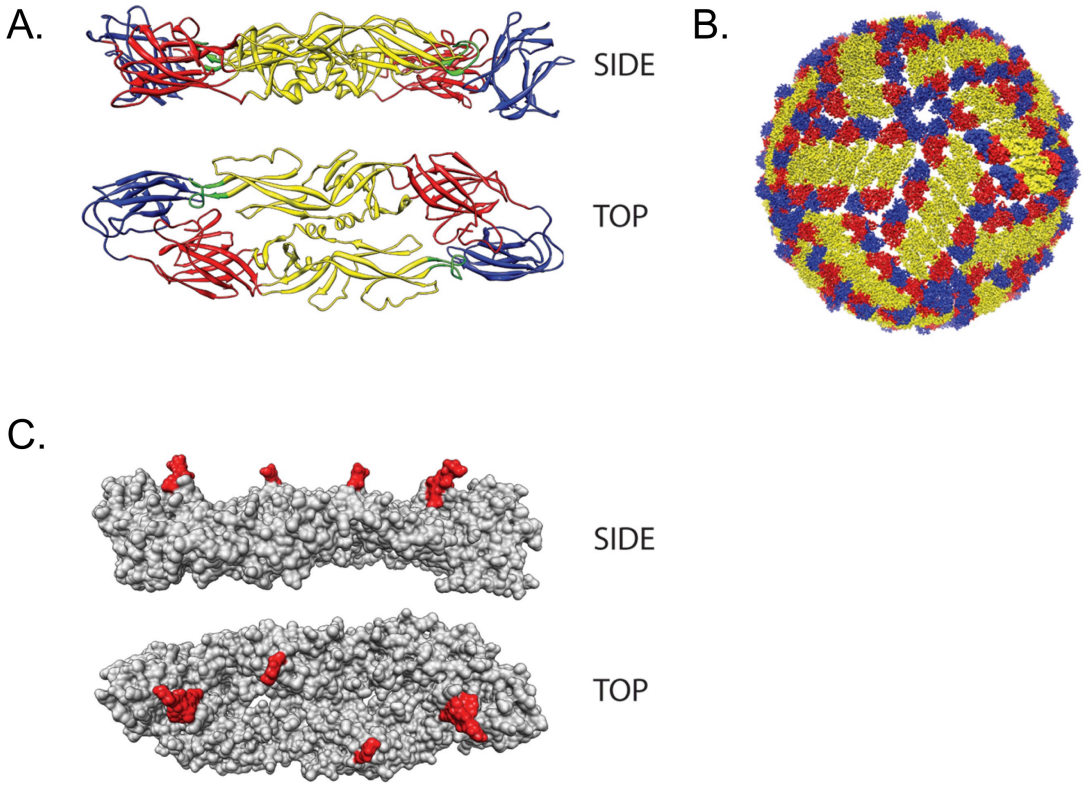


Figure 2.3 Structure of the flavivirus E protein. (**A**) Structure of the ectodomain of the flavivirus E protein dimer (PDB: 1OAN) from a side view (top panel) and top view (bottom panel). Domains I, II, and III are shown in red, yellow, and blue, respectively. The fusion loop in domain II is shown in green. (**B**) Structure of a mature flavivirus virion (PDB: 4CCT). The E proteins are arranged as 90 antiparallel homodimers that densely coat the virion surface. (**C**) The two possible glycans on the E protein are highlighted in red in the E protein dimer (PDB: 1OAN) from a side view (top panel) and top view (bottom panel).

have also been identified, but they have limited neutralization potential and display cell type-dependent patterns of activity (S. Mukherjee and T. Pierson, unpublished data) (50, 59-61).

In addition to virions, subviral particles (SVPs) lacking a nucleocapsid are also produced from flavivirus infected cells. SVPs are smaller (~30nm in diameter) than virions and have a different symmetry and organization of their E proteins, though SVPs exhibit fusogenic properties similar to that of virions (62, 63). In contrast to the 180 E proteins packaged into virions, SVPs contain 60 E proteins organized into 30 dimers in T=1 icosahedral symmetry (62). The differences in the structural organization of virions and SVPs results in differential recognition by antibodies, indicating the influence of quaternary structure and epitope display on antibody recognition of flaviviruses (64).

2.1.5 Virus entry

The E proteins orchestrate the entry of flaviviruses into target cells, which occurs through receptor-mediated endocytosis, followed by pH-dependent fusion in endosomes (65). Cellular factors that mediate virus entry are not completely defined, though attachment factors that enhance virion binding to cells have been identified, including C-type lectins DC-SIGN and DC-SIGNR, mannose receptor, heparin sulfate, and phosphatidylserine receptors of the TIM and TAM protein families (31, 66-70). The role these molecules play in the cell biology of virus entry is incompletely understood, and may extend beyond simply facilitating virus attachment. To date, cellular proteins required for the low-pH-mediated conformational change in the virus have not been identified; flaviviruses are

capable of fusing directly with synthetic membrane preparations (71, 72). Single particle tracking studies suggest virus entry occurs within 17 minutes of stable attachment of the virion (73). *In vitro* studies clearly demonstrate that viral fusion occurs very rapidly (within seconds) after exposure to mildly acidic conditions (55, 71, 72).

2.2 Dengue virus

2.2.1 Diversity

The DENV serocomplex is divided into four serotypes that share between ~63-77% amino acid identity of their E glycoproteins. Despite the genetic differences, the four DENV serotypes display similar clinical manifestations and epidemiological features. Viruses within a DENV serotype share over 90% amino acid identity. Each serotype is further divided into three to five distinct genotypes, with some evidence linking virus genotype to disease severity (74, 75).

2.2.2 Distribution

Together, the dengue viruses are the leading cause of mosquito-borne viral disease in the world, with an estimated 390 million infections occurring annually, of which 96 million are apparent infections, and 294 are inapparent infections (mild or asymptomatic) (1). Due to the widespread distribution of its primary mosquito vector, *Aedes aegypti*, DENV infections occur in countries throughout tropical and subtropical regions in Asia, Africa, Australia, and the Americas. Asia bears the highest dengue burden, where around 70% of apparent dengue infections occur (1). Populations in DENV-endemic areas can display

very high rates of seroprevalence. A study in the Ratchaburi Province of Thailand found 97% of pregnant women had detectable antibodies against DENV (76). Of concern, there has been an increase in the frequency and magnitude of DENV epidemics in the past several decades, likely due to factors such as increased urbanization, globalization, climate change, and vector spread, with predictions that DENV will continue to spread (77). There has also been an increase in the co-circulation of serotypes, with many DENV-endemic countries having reported detection of more than one, or in some cases up to all four DENV serotypes (78). DENV is additionally maintained in a sylvatic cycle between *Aedes* mosquitoes and nonhuman primates, with the potential for spillover into the human population (79).

2.2.3 Pathogenesis

Dengue disease typically manifests as dengue fever (DF), an acute but incapacitating febrile illness with symptoms that may include maculopapular rash, headache, myalgia, arthralgia, retro-orbital pain, and thrombocytopenia (80). DF is typically self-limiting and lasts about one week. However, DENV infection may also result in severe disease such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), which are characterized by hemorrhagic manifestations and plasma leakage. While rare (~0.5% of symptomatic cases), these disease manifestations have the potential to be fatal (1, 80).

The causes of DHF/DSS are not fully understood, but aspects of the host immune response have been implicated, particularly during secondary DENV infections. Primary DENV infection is thought to induce a type-specific immunity that offers long-term

protection only against the infecting serotype. Following a brief period of cross-protection, individuals are susceptible to a secondary infection with a heterologous serotype (11). Secondary, heterotypic infections are associated with significantly higher rates of severe disease relative to primary infections (4-7). This enhancement of disease during secondary infections is likely mediated by the cross-reactive but non-protective immune response generated during the primary infection against heterologous serotypes. Antibodies have been implicated in DENV pathogenesis through a mechanism called antibody-dependent enhancement of disease (ADE), in which non-neutralizing antibodies or sub-neutralizing concentrations of antibodies bind the virus and enhance virus uptake into cells expressing Fc-gamma receptors (FcγR) such as monocytes, macrophages and dendritic cells (81, 82). ADE has been extensively studied *in vitro*, though the mechanisms by which ADE results in severe disease *in vivo* are not fully understood. Increased viremia and altered production of cytokines and other inflammatory mediators have been suggested as possible mechanisms (83). The ADE model is supported by observations that higher rates of DHF/DSS during primary infection occur in infants born to DENV-experienced mothers. Specifically, enhanced disease in infants is frequently observed several months after birth, when maternally acquired antibodies have waned to levels that are sub-neutralizing and capable of mediating ADE *in vitro* (79, 84).

Cellular immune responses have also been implicated in severe disease, potentially as a result of cross-reactive T cell responses that lead to aberrant cytokine production (85). Determinants of disease severity may also include viral-specific factors. For example, while all four serotypes can cause severe disease during primary or secondary infections,

severe disease during secondary infections has a higher association with the DENV2 serotype (6, 86, 87). Specific genotypes within serotypes have also been associated with more severe disease (88).

2.3 Humoral Immune Response

A principal component of the humoral immune response is the repertoire of antibody molecules secreted by B lymphocytes. Antibodies are Y-shaped glycoproteins composed of two identical heavy chain/light chain heterodimers linked by disulfide bonds (reviewed by (89)). The arms of antibody molecules (or Fabs) are connected to the remainder of the protein by flexible hinges, which diversify the angles with which antibodies may bind antigens. The distal end of each arm forms the antigen-binding site of the molecule, called the variable (V) region. Both heavy and light chains contain three hypervariable loops (called complementarity determining regions, CDR) that come together to form the V structural region. The constant (Fc) portion of the antibody is modified by an N-linked oligosaccharide that contributes to interactions with molecules and cells of the immune system to mediate a range of effector functions (discussed below).

2.3.1 Diversity Of The Humoral Immune Response

The humoral response is capable of producing an incredibly diverse repertoire of antibody molecules with unique antigen binding properties. In part, this diversity is encoded directly by the germline. Genes encoding the variable heavy (V_H) and light (V_L) chains exist as multiple gene segments. The heavy chain is encoded by multiple variable

(V), joining (J), and diversity (D) gene segments. Two chromosomes encode the V and J gene segments that form the light chain (the κ and λ loci). Full length antibody molecules are assembled from these gene segments in developing B lymphocytes by a process called V(D)J recombination (reviewed by (90)). This mechanism creates combinatorial diversity through the random pairing of VDJ gene segments (at the V_H loci) or VJ gene segments (at the V_κ or V_λ loci) during somatic gene rearrangement. Additional diversity arises during V(D)J recombination because nucleotides are introduced or deleted at the junction of segments as they are linked together. The recombined variable region of the heavy chain is then joined to μ and δ constant gene segments (to make IgM or IgD antibody subclasses, respectively). Random pairing of heavy and light chains results in formation of an intact antibody molecule (and B cell receptor (BCR)). The process of allelic exclusion ensures each lymphocyte produces only a single antibody molecule (reviewed in (91)).

2.3.2 Germinal center formation and affinity maturation

The antibody repertoire produced by B-lymphocytes is refined and diversified further upon exposure to antigen. With appropriate T cell help, B cell recognition of an antigen results in cellular activation, extensive proliferation, and potentially Ig class switching, during which the Fc portion of the antibody gene can be exchanged for another with different functional properties. Antigen-primed B cells can develop into short-lived plasma cells (PCs), which are terminally differentiated cells characterized by high antibody secretion and low BCR expression. PCs and their proliferating precursors, plasmablasts, are responsible for the production of the early antibody response (**Figure**

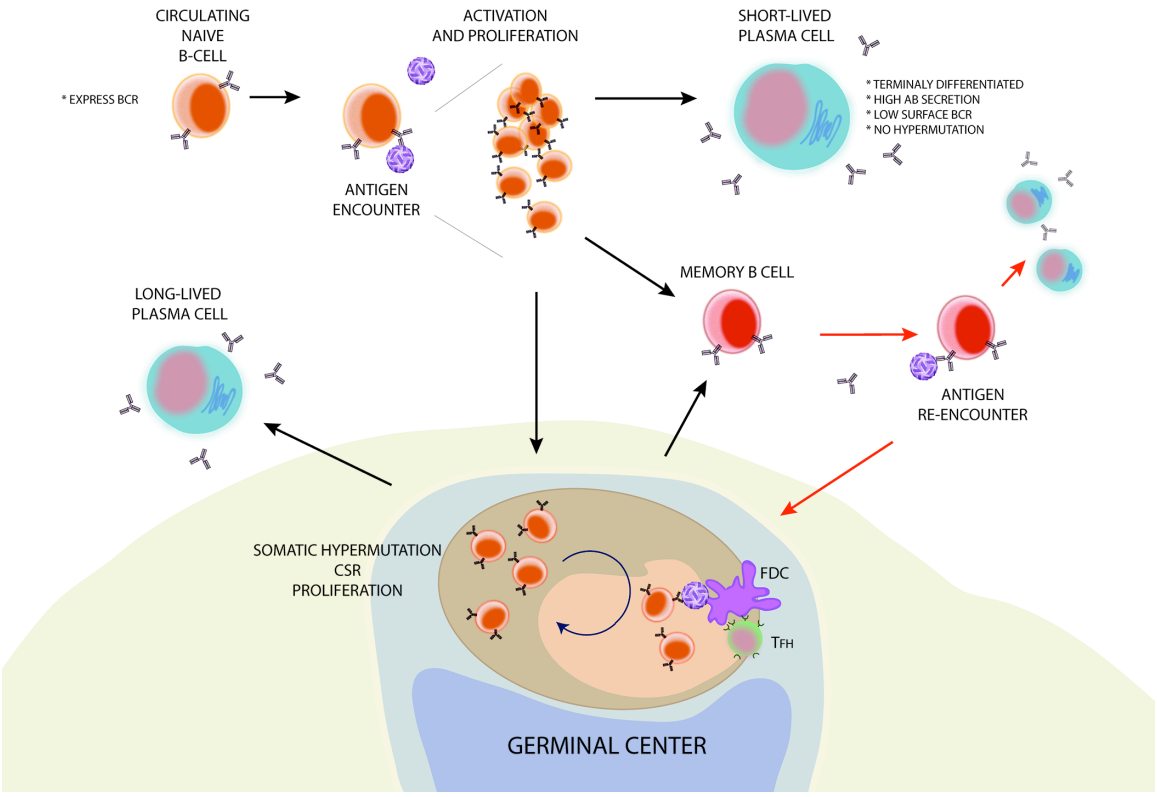
2.4). Antigen-primed B cells can also participate in the formation of germinal centers (GCs), along with follicular helper T cells (T_{FH}) and follicular dendritic cells (FDC). GC reactions result in the production of long-lived plasma cells and memory B cells (discussed further below) (92), although memory B cells may also arise from GC-independent mechanisms (93).

In GCs, B cells refine the antibody response via the process of affinity maturation. Affinity maturation occurs through iterative rounds of somatic hypermutation, during which point mutations are introduced into the antibody V regions, followed by T_{FH} cell-based selection of clones with the highest antibody affinity. One study estimated that the average number of mutations in the V_H region of IgG among memory B cells and germinal center cells was 14, with 88% of the sequenced V_H genes encoding between three and 29 mutations (94). GCs are sites of competition among B cell clones for T cell help. After multiple rounds of affinity-based selection, GCs may undergo "monoclonalization", with one high-affinity B cell clone beginning to dominate any given mature GC (reviewed by (95)). In GCs, T_{FH} cells also signal B cells to initiate class switching (96-98).

2.3.3 Effector Functions Of Antibodies

While the Fab region of an antibody defines its specificity, the invariant Fc portion of the heavy chain determines its effector function. The antibody class switching mechanism of B cells has the potential to create antibodies of similar specificity capable of orchestrating diverse immune responses. The Fc region of antibody heavy chains interacts with Fc

Figure 2.4 Sources of antibodies. Upon naïve B cell recognition of an antigen and activation by a cognate T cell, activated B cells undergo extensive proliferation. Activated B cells can then follow one of several paths: (i) they may terminally differentiate into short-lived plasma cells (PCs) which have low surface Ig levels and high Ig secretion rates; (ii) they may differentiate into memory B cells (MBCs) which retain BCR expression but do not constitutively secrete antibody; (iii) they can participate in the formation of germinal centers (GCs), along with follicular helper T (T_{FH}) cells and follicular dendritic cells. In GCs, B cells undergo rapid proliferation, further diversification of their antibody gene through somatic hypermutation, and class-switch recombination (CSR) during which the Fc region of the Ig gene may be exchanged for another to modulate antibody effector function. Selected GC B cells receive signals to differentiate into PCs or MBCs; other GC B cells undergo apoptosis. PCs may be short-lived and remain in the lymphoid organs, or become long-lived plasma cells (LLPCs) and migrate to the bone marrow, where they will continue to secrete antibody independent of the presence of antigen. LLPCs are most likely responsible for the long-lived, pathogen-specific antibody titers in serum that can last years or decades following infection or vaccination. Distinct from LLPCs, MBCs are long-lived cells that remain in circulation and peripheral lymphoid tissue. Through expression of their BCR, they can be re-activated by antigen. Upon re-stimulation, they may set up germinal centers, undergo further somatic hypermutation and class switching, and differentiate into antibody-secreting plasmablasts and PCs.


CIRCULATING
NAIVE
B-CELL
* EXPRESS BCR
ACTIVATION
AND PROLIFERATION
ANTIGEN
ENCOUNTER
SHORT-LIVED
PLASMA CELL
* TERMINALY DIFFERENTIATED
* HIGH AB SECRETION
* LOW SURFACE BCR
* NO HYPERMUTATION
MEMORY B CELL
ANTIGEN
RE-ENCOUNTER
LONG-LIVED
PLASMA CELL
SOMATIC HYPERMUTATION
CSR
PROLIFERATION
FDC
TFH
GERMINAL CENTER

receptors (FcR) on immune effector cells or soluble immune molecules such as those in the complement system (89). The strength of these interactions varies among antibody class and can be influenced by the particular carbohydrate modification on the antibody molecule (99, 100). All IgG subclasses encode an N-linked glycosylation site at residue 297 of the heavy chain. While the position of the glycosylation site is conserved, the composition and structure of the oligosaccharide added to the antibody is influenced by the host immune activation state (101, 102). This provides the immune system with an ability to tune antibody effector function beyond class switch recombination during the course of the immune response. For example, the IgG repertoire of HIV-infected individuals is modified by sugars with an agalactosylated, pro-inflammatory glycan profile as compared to uninfected individuals; this is particularly pronounced in elite controllers of HIV (103). Of interest, changes in the carbohydrate profiles of HIV-1 reactive and non-reactive antibodies differed within individuals. This skewing towards agalactosylated antibodies in infected individuals and elite controllers correlated with increased antibody-dependent cellular viral inhibition (ADCVI) activity in vitro.

The antibody heavy chain orchestrates antibody effector functions including antibody-dependent cellular cytotoxicity (ADCC), opsonization, mast cell activation, and complement activation. Thus, non-neutralizing antibodies elicited by immunization or infection may offer protection from viral infections through effector functions mediated by the Fc region. Studies in murine models of both WNV and HIV-1 infection have demonstrated the importance of the Fc effector functions of antibodies in mediating protection *in vivo* (104-107). For example, anti-WNV mAbs with poor *in vitro*

neutralizing ability can mediate *in vivo* protection in mice, but eliminating IgG binding to complement component C1q and Fcγ receptors via removal of the N-linked glycan at N297 eliminated their protective capacity (104). Conversely, the inability of strongly neutralizing mAbs with mutations at N297 to interact with Fcγ-receptors may be exploited for the development of DENV therapeutics, as these non-glycosylated nAbs do not support (and may competitively inhibit) the antibody dependent enhancement processes thought to contribute to severe disease outcomes (108, 109).

2.3.4 Where Do Antiviral Antibodies Come From?

Long-term humoral immunity results from at least two distinct cell populations (**Figure 2.4**). Long-lived plasma cells (LLPCs) constitutively secrete antibody independent of the presence of antigen, and are most likely responsible for the long-lived, pathogen-specific antibody titers in serum that can last years or decades following infection or vaccination (110, 111). LLPCs reside predominantly in the bone marrow and are terminally differentiated. They do not possess antigen receptors and are not reactivated upon antigen re-exposure. In contrast, memory B cells (MBCs) remain in circulation and peripheral lymphoid tissue where they may re-encounter antigen (112). MBCs express BCR on their surface, but do not constitutively secrete antibody. Upon re-stimulation by antigen, MBCs may differentiate into antibody-secreting plasma cells, and may form germinal centers to undergo further affinity maturation and class switching (113). MBCs are responsible for the anamnestic antibody response that occurs upon secondary exposure to an antigen, responding more rapidly and in greater magnitude to antigenic stimulation than their naïve predecessors.

LLPCs and MBCs differ with respect to the extent of the affinity maturation of the antibodies they express (114). Only B cells capable of producing antibodies with high affinity for the antigen are selected for LLPC formation and persistent antibody production, whereas B cells that produce antibodies with lower affinity for antigen may survive as MBCs (114, 115). Antibodies from these two compartments may differentially contribute to protection from infection. A study by Purtha *et al*. (116) demonstrated that, following infection of mice with WNV, antibodies from MBCs were able to recognize not only the infecting strain of WNV but also a variant encoding a mutation in a known neutralizing antibody epitope. In contrast, the LLPC antibody response was specific for the infecting strain. Thus, while LLPC-derived antibody was of higher affinity and capable of conferring immediate protection against homotypic viral reinfection, the MBC compartment may be critical for the recognition of a genetically diverse challenge.

2.4 Antibody-mediated neutralization

Antibody-mediated neutralization is defined as direct inhibition of viral infectivity that occurs as a result of antibody binding to the virus particle. How antibodies block infection has been studied intensely for decades (reviewed by (117, 118)). Because nAbs have great potential as antiviral therapeutics, and often correlate with protection following vaccination (119), structural and molecular insights into mechanisms of neutralization have considerable translational value. Recent technical advances that enable the isolation and study of human monoclonal antibodies have not only accelerated progress towards therapeutics and diagnostics, but have also provided an important

reference to guide reductionist mechanistic studies towards antibodies with the most relevant specificities *in vivo* (120, 121).

2.4.1 Neutralization By The Numbers

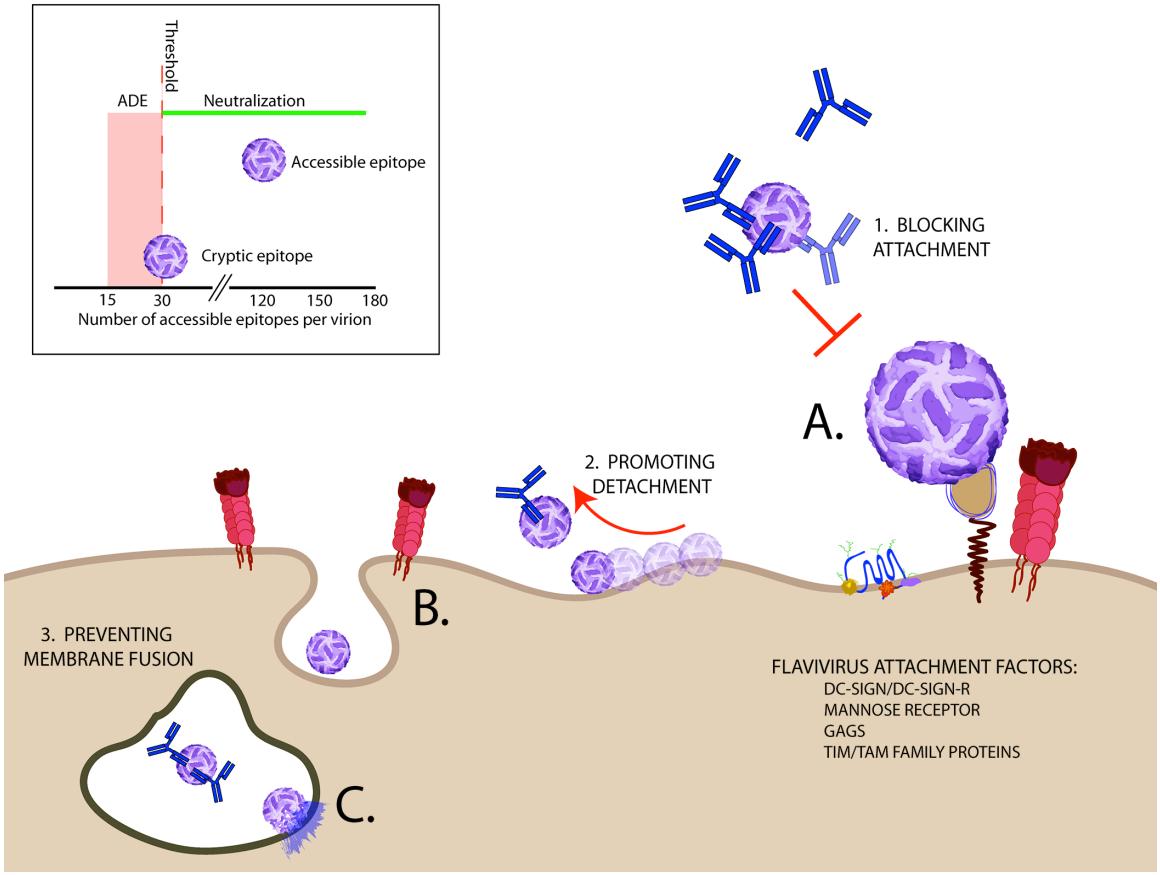
How many antibodies are required to neutralize a virus? The stoichiometry of antibody-mediated neutralization has been intensely debated (reviewed in (122, 123)). A "single hit" model suggests that one antibody molecule binding to a virus particle in the right location is sufficient to inactivate the virion. This hypothesis has been supported largely by kinetic arguments in studies of poliovirus, Western equine encephalitis virus, and influenza (124, 125). However, limitations of these kinetics arguments have been reviewed (118). An alternative "multiple-hit" model proposes that neutralization of an individual virus particle requires engagement by numerous antibody molecules (122). An interesting extension of this model suggested that the number of antibodies necessary for neutralization was a reflection of the size of the virion because of a requirement to fully occlude its surface (reviewed by (123)). Estimates of the stoichiometry of neutralization have been determined for multiple viruses, including phage MS2 (126), poliovirus (127-129), WNV (130), papillomavirus (131), influenza virus A (125), and rabies virus (132, 133). While in many cases the neutralization threshold for structurally distinct groups of viruses does indeed correlate positively with virion size in agreement with the "coating theory" (123), factors that determine the number of antibodies required for neutralization are not well understood. For example, the small number of functional trimers on the HIV-1 surface allows for neutralization with a stoichiometry much lower than predicted for a virion of that size (134, 135). For WNV, genetic and biochemical studies using mAbs

that bind an epitope on the lateral ridge of DIII suggest that the binding of 30 mAbs to the virion is required for neutralization (**Figure 2.5**, inset) (130, 136). How the stoichiometric requirements for neutralization of flaviviruses differ among epitopes or neutralization mechanisms (see section below) is unknown (137). In support of this caveat, the number of antibodies required to neutralize infection via different mechanisms has been suggested to differ (58, 138). At least two factors have been shown to govern how many antibodies may bind the virion at any given concentration of antibody (reviewed by (137)). Antibody affinity determines the fraction of viral epitopes bound in the presence of a particular mAb concentration. More critically, the accessibility of epitopes on the intact infectious virion provides the "denominator" for this relationship: epitope accessibility ultimately governs the number of antibodies capable of binding at saturation. Not all epitopes are equally accessible for binding (**Figure 2.5**, inset), and many factors with the potential to impact epitope accessibility have been described (for flaviviruses, see (137)). Factors that reduce epitope accessibility may make viruses less sensitive to neutralization, and thus have the potential to contribute to immune evasion, as detailed below. Further studies to correlate epitope location, neutralization, and the occupancy requirements for neutralization are warranted.

2.4.2 Mechanisms Of Neutralization

Antibodies have the ability to block viral infection at any number of steps in the process of viral entry (reviewed by (118)). These include viral attachment to the cell surface, viral interactions with receptors or co-receptors, fusion with host membrane (for enveloped viruses), membrane penetration or genome injection (for non-enveloped viruses), or viral

Figure 2.5 Flavivirus entry and mechanisms of antibody-mediated neutralization. (**A**) Flavivirus entry occurs following viral interaction with attachment factors such as C-type lections DC-SIGN and DC-SIGNR, mannose receptor, heparin sulfate, and phosphatidylserine receptors of the TIM and TAM protein families. (**B**) Following virus attachment, flaviviruses undergo clathrin-mediated endocytosis. (**C**) They can then enter the cytoplasm by pH-dependent fusion in the endosome. Antibody-mediated neutralization of flaviviruses may be achieved by inhibiting virus infectivity at a number of viral entry steps such as (i) preventing virus attachment to the cell surface, (ii) promoting virus detachment from cells, and (iii) inhibiting viral fusion with endosomal membranes. Inset: Neutralization occurs when antibodies bind flaviviruses with a stoichiometry that exceeds a particular threshold (130). Antibody-dependent enhancement of infection (ADE) can occur if the number of antibodies bound to the virion does not reach the stoichiometric threshold for neutralization. The number of antibodies bound per virion is modulated by antibody affinity as well as by epitope accessibility. Therefore, antibodies that bind cryptic epitopes that are poorly accessible for antibody recognition may not be able to achieve a stoichiometry sufficient to exceed the threshold requirements for neutralization, despite high affinity for the epitope. In contrast, antibodies that bind highly accessible epitopes can exceed the stoichiometric threshold for neutralization at low occupancy.


Threshold
ADE
Neutralization
Accessible epitope
Cryptic epitope
15
30
120
150
180
Number of accessible epitopes per virion
1. BLOCKING ATTACHMENT
A.
2. PROMOTING DETACHMENT
B.
3. PREVENTING MEMBRANE FUSION
C.
FLAVIVIRUS ATTACHMENT FACTORS:
DC-SIGN/DC-SIGN-R
MANNOSE RECEPTOR
GAGS
TIM/TAM FAMILY PROTEINS

genome uncoating (**Figure 2.5**). Blocking cell surface attachment or receptor engagement through steric hindrance may be a common mechanism, and has been suggested to explain the activity of DENV-immune sera (139). However, many antibodies have been shown to be capable of blocking infection at a post-attachment step (48, 56, 140-143). These nAbs may inhibit conformational changes of a viral protein required to mediate virus entry. For example, the WNV-specific mAb E16 has been shown structurally to trap E proteins in a radially extended intermediate at low pH (144). Like E16, the mAb CR4354 inhibits WNV at a post-attachment step and can inhibit viral fusion with synthetic liposomes (56, 145). The structure of CR4354 Fab bound to WNV revealed a discontinuous epitope that spanned neighboring E proteins, suggesting this mAb and others that bind complex quaternary epitopes might block fusion by crosslinking E proteins on the virion (145). While the multiple-hit hypothesis assumes neutralization is a reversible process (146), in some cases, antibody binding results in an irreversible change in virion infectivity that persists upon reversal of binding. Anti-HIV mAbs that bind the membrane-proximal external region (MPER) on gp41 induce shedding of viral glycoprotein gp120 from the virion, which renders virions non-infectious even when antibody binding is reversed (147).

While Fab fragments of neutralizing antibodies can block infection (117), the Fc region of antibodies is also likely important for neutralization. While the surface area buried by an epitope/paratope interaction is relatively small, generally less than 900 $Å^2$ (148), the intact antibody molecule has the potential to occupy a very large area due to its structural flexibility. Thus, the antiviral activity of antibodies likely reflects the contribution of the

entire molecule, which has the potential to not only influence the number of antibody molecules docked on the virion (relative to Fab fragments), but also interfere with many processes that occur during viral entry (64, 149). For example, the orientation of the Fc region of virus-bound Abs has been hypothesized to control the number of WNV DIII-reactive mAb E33 molecules docked to viruses of different maturation states (150). Thus the large size of the antibody molecule has the potential to influence the accessibility of surrounding epitopes and thereby impact conditions that support antibody-mediated neutralization.

2.5 Viral Evasion Of Antibody-Mediated Neutralization

2.5.1 Sequence Variation And Antigenic Diversity

Viral genomes mutate at a relatively rapid rate, allowing the selection of mutations within epitopes recognized by antibodies (151, 152). Viruses that can tolerate a large number of mutations in their structural proteins, such as HIV, hepatitis C virus (HCV), influenza virus, and noroviruses allow for rapid and substantial antigenic drift in the presence of immune pressure (121, 153-156). This is evident in the yearly requirement for reformulation of the seasonal influenza vaccine, as influenza viruses are extremely adept at acquiring mutations that aid in antibody-escape (157, 158). For viruses that cause chronic infections, intra-host generation of antigenic diversity is observed over time as new viral variants emerge to escape the nAbs generated early in the immune response. As the antibody response evolves to recognize poorly neutralized variants, new viral variants emerge to escape those antibodies. This iterative selection of neutralization-escape variants constantly elicits the production of new antibodies (159-161). However, such

rapid antigenic evolution is not apparent for all viruses, even among RNA viruses with error-prone RNA polymerases. The monovalent YFV vaccine 17D has been in use since the 1950s. Due to a lack of significant antigenic drift, there has not been a need to reformulate the YFV vaccine, even after 60+ years of use (162). Many factors could limit the impact of the humoral immune response on shaping viral evolution, such as differences in viral transmission, replication, or pathogenesis, and the existence of viral reservoirs.

2.5.2 Conformational Masking Of Conserved Regions

Conserved structural features may play important functions in the viral life cycle and may be less tolerant to sequence variation and thus represent a site of vulnerability to antibodies. Many viruses have therefore evolved mechanisms to conceal conserved regions of their structural proteins. For example, the "canyon hypothesis" speculates that the receptor binding domain (RBD) of some picornaviruses is buried in a canyon on the surface of the viral capsid in order to protect it from immune recognition (163), although this strategy is imperfect (164). Likewise, the HIV-1 gp120 RBDs are positioned in recessed pockets of the envelope spike that are not accessible for recognition by most antibodies (165, 166). The DII-FL of flaviviruses is critical for mediating fusion during viral entry, is highly conserved among distantly related flaviviruses, and frequently targeted by the antibody response (discussed below) (167-169). However, antibodies with this specificity are typically characterized by limited neutralizing activity because of the poor exposure of the fusion loop epitope, which is obscured by its proximity to residues

of the opposing E protein on the structure of the mature virion (54, 170). Numerous other examples of cryptic flavivirus epitopes have been reported (138, 171-173).

2.5.3 Regulation of epitope accessibility by flaviviruses

At least two factors have the potential to modulate the accessibility of epitopes on flaviviruses. While steric constraints limit antibody accessibility on the mature virion, inefficient maturation of flaviviruses results in the release of partially mature virions on which epitope accessibility may differ (32, 33, 35, 170). Decreasing the efficiency of virion maturation results in an increase in neutralization sensitivity for many classes of antibodies (32). The conformational dynamics of E proteins incorporated into the virion also modulates epitope accessibility. Viral "breathing" has been shown in several systems to impact neutralization sensitivity (138, 174-177). Antibodies have the potential to trap transiently exposed structures as they appear in the ensemble of structures sampled by flaviviruses at steady state. Recent studies have demonstrated that the reversible exposure of cryptic epitopes by WNV and DENV contribute to time-dependent patterns of neutralization (138, 178). Similar observations have been made with antibodies to internal components of the capsid of some picornaviruses (174). Conformational dynamics of Env may also impact epitope exposure and neutralization sensitivity of HIV-1. When the conformational dynamics of two HIV strains were compared by single-molecule fluorescence resonance energy transfer (smFRET), the laboratory-adapted, neutralization-sensitive strain demonstrated increased dynamics and transitioned more frequently between closed and open conformational states compared to the clinically isolated, neutralization-resistant strain (179).

2.5.4 Low Density Of Surface Glycoproteins

As mentioned, the dense arrangement of flavivirus E proteins may impose steric constrains on antibody epitope accessibility. However, viruses may also evade neutralization by decreasing the number of structural proteins displayed on their surface. A reduction in the number of surface glycoproteins that can be targeted by nAbs may prevent antibody engagement from exceeding the stoichiometric threshold required for neutralization. Human cytomegalovirus (HCMV) has been shown to reduce incorporation of the surface glycoprotein gH under selective pressure by antibody *in vitro*, resulting in a resistance to neutralization by gH-specific mAbs (180). A relatively low density of structural proteins on the surface of virions may also prevent bivalent engagement of the virus particle by antibody, which in turn limits antibody avidity. This has been suggested to limit the neutralizing activity of some HIV-reactive mAbs (reviewed by (181)). Biochemical and structural studies have estimated that the average HIV virion has a small number of Env spikes on its surface (<15) (182-184). Comparisons of the neutralization potency of IgG and Fab fragments of anti-HIV antibodies revealed similar potencies, suggesting bivalent recognition is uncommon (181). However, elegant studies by Björkman and colleagues with engineered antibody-based molecules capable of intra-spike bivalent binding demonstrate greatly increased neutralization potencies for antibodies with certain epitope specificities (185). While the densely packed, pseudo-icosahedral arrangement of E proteins on flavivirus virions may allow bivalent engagement by antibodies, surprisingly only one anti-flavivirus mAb, the DENV DIII-

specific mAb E106, has been characterized to date that requires bivalent binding for its neutralizing activity (186).

2.5.5 Glycan Shields

Although flavivirus E proteins contain only a limited number of glycans (1-2), many viral structural proteins contain multiple glycosylation sites, which can mediate immune evasion (reviewed by (187)). The presence of N- and O-linked glycans may lower the immunogenicity of particular regions of viral glycoproteins, as carbohydrate structures on virions may be recognized as "self" by the immune system (166). Several viruses, including HIV-1, Ebola virus, and HCV, utilize a 'glycan shield' to avoid antibody recognition (159, 188). For example, the HCV envelope glycoproteins E1 and E2 together have up to 16 N-linked glycosylation sites, most of which are highly conserved. Several of the glycans on the E2 glycoprotein have been shown to influence the susceptibility of HCV virions to neutralization (188). Additionally, changes in the composition of a glycan shield provide a mechanism to rapidly evolve in response to immune pressure. Alterations in the number, placement, and type of glycans on HIV-1 and simian immunodeficiency virus gp120 occur in order to mask epitopes recognized by nAbs (159, 189, 190). How the E protein glycosylation status of flaviviruses affects antibody recognition is not well understood, though antibodies that make contacts with both glycans on DENV have been reported (191).

2.6 The Humoral Immune Response to DENV

Several lines of evidence suggest the important role that the humoral response plays in protection against DENV. Antibody responses serve as correlates of protection for vaccines against related flaviviruses, including yellow fever virus (YFV), Japanese encephalitis virus (JEV), and tick-borne encephalitis virus (TBEV) (192-195). Cohort studies in DENV-endemic areas have observed that pre-existing homotypic DENV nAb titers correlate with protection from DENV infection (196). Additionally, in animal models for several flaviviruses including for DENV, passive transfer of mAbs can mediate protection from infection (47, 48, 142, 197-199). However, antibodies also have the potential to be pathogenic during DENV infection through ADE, complicating our understanding of their role in mediating protection, and our ability to exploit them for the development of anti-DENV vaccines and therapeutics.

In addition to the humoral immune response, the cellular immune response may also contribute to protection from DENV infection, though its role is not completely understood. In mouse models, immunization with DENV-derived $CD4^+$ or $CD8^+$ T cell epitopes enhances host control of infection following challenge with DENV (200, 201). T cell depletion and adoptive transfer experiments have further suggested a role for T cells in contributing to protection from DENV infection in mice (201-204). In humans, HLA alleles have ben associated with DENV disease severity (205-211). One comprehensive study of $CD8^+$ T cell responses in DENV-endemic population additionally revealed an association between HLA alleles and the magnitude of the T cell response. Certain HLA class I alleles in the population were associated with weak $CD8^+$ T cell responses and

increased risk of severe disease, while other alleles correlated with strong, multi-functional T cell responses and protection from severe disease (212). This study further demonstrated that the majority of the $CD8^+$ T cell response is directed against epitopes on nonstructural proteins 3, 4B, and 5, suggesting that the inclusion of these proteins may be advantageous for a DENV vaccine.

2.6.1 The Antibody Response to Primary DENV Infection

Following primary DENV infection, anti-DENV IgM appears within three to eight days of the onset of illness (213). The class-switched IgG subsequently appears 10-15 days after the onset of symptoms, and is predominantly made up of IgG1 and IgG3. The IgM response is short-lived and declines to undetectable levels in most patients within 60 days following infection (214). In contrast, the IgG response is long-lived, with IgG detectable by both ELISA and plaque reduction neutralization test (PRNT) more than 60 years after infection (9).

The antibody response elicited by a primary DENV infection typically exhibits cross-reactivity against heterologous serotypes, but potent neutralization only against the homologous serotype (57). The individual antibodies that constitute a polyclonal antibody response to DENV vary with respect to their specificity, breadth, and potency (47-49, 215, 216). While the potently neutralizing fraction of the antibody response following primary infection or vaccination is predominantly specific for the infection serotype (type-specific), the majority of antibodies isolated from DENV-infected humans appear to be cross-reactive and weakly neutralizing (50, 57, 59). The type-specific

response is thought to offer long-lasting protection against homotypic reinfection with the homologous serotype, while the cross-reactive response appears to induce a short-lived period of cross-protection, but likely wanes below protective levels, leaving DENV-experienced individuals susceptible to secondary, heterotypic infections with the remaining three serotypes (11). As the cross-reactive antibody response wanes below protective levels, it may contribute to DENV pathogenesis during secondary, heterotypic infections through the mechanism of ADE.

2.6.2 The Antibody Response to Secondary DENV Infection

During secondary DENV infections, there is a rapid and potent proliferation of cross-reactive plasmablasts that predominantly secrete IgG (217). The antibody response following secondary DENV infections is characterized by increased cross-reactivity to heterologous serotypes relative to primary infections (218, 219). The cross-reactive response elicited by secondary heterotypic infections appears to offer protection against the remaining two serotypes, as the likelihood of developing DF during a post-secondary infection (3° or 4°) is significantly reduced relative to primary and secondary infections (220). Hospitalization for 3° or 4° DENV infections is rare. However, post-secondary cases that do result in clinically apparent disease may be just as likely to lead to DHF as secondary infections are (221).

2.6.3 The Antibody Response to DENV Vaccination

Though no DENV vaccine is currently licensed, numerous candidates have advanced to testing in clinical trials, including several live-attenuated vaccines, a recombinant E protein vaccine, a purified inactivated virus vaccine, and a DNA vaccine (reviewed by (222)). The most clinically advanced vaccine candidate is a live-attenuated, tetravalent vaccine (CYD-TDV) from Sanofi Pasteur that has been tested for efficacy in a phase IIb trial and in two phase III trials (223-225). CYD-TDV is a chimeric vaccine that utilizes the YFV vaccine strain 17D as a backbone, but incorporates structural genes (C, prM, and E) derived from each of the four DENV serotypes. CYD-TDV displayed modest protective efficacy in phase III trials, with overall efficacies of 61% and 67% in the Latin American and Asian trials, respectively. Efficacy studies were performed in school children in DENV-endemic areas in Asia and Latin America that had high seropositivity rates (~70-80%) before the administration of the vaccine. While efficacy was high (74-84%) in vaccinees that were seropositive before being vaccinated due to prior exposure to DENV, vaccine efficacy was low (35-42%) in individuals that were DENV-naïve before vaccination, demonstrating the vaccine's limited ability to prime a novel immune response (223, 225). Interestingly, despite the fact that CYD-TDV induced balanced neutralizing antibodies against all four serotypes *in vitro*, vaccine efficacy was not uniform among the serotypes. Efficacy against DENV3 and DENV4 ranged from 74-78%, efficacy against DENV1 was 50%, and low efficacy was observed for DENV2 (35-52%) (223, 225).

2.7 Remaining questions

The observation that CYD-TDV elicited a neutralizing antibody response yet was not protective against DENV2 infection highlights the need for a better understanding of the immune response to DENV. Notably, the neutralization titers against DENV2 were comparable to those against the other serotypes (223). What type of immune response is protective against DENV and how can we measure it? Is the immune response that mediates protection different for DENV2 than for other serotypes? Are neutralizing antibodies a correlate of protection for DENV? Is the current plaque-reduction neutralization test (PRNT) a valid test for assessing the antibody response? Is there a qualitative or functional difference between antibodies elicited following natural infection versus those elicited following tetravalent vaccination? Notably, CYD-TDV contains only structural genes from DENV; the nonstructural genes are from the YFV backbone. Are the non-structural proteins important for immune recognition?

The increased risk of severe disease in DENV-sensitized populations significantly complicates vaccine development. In order to avoid the potential for vaccine-mediated enhanced disease, a vaccine must confer protection against all four DENV serotypes. Therefore, it is crucial to understand the humoral immune response to DENV not only in the context of individual and sequential infections, but also in the context of multivalent vaccination. While primary infection with one serotype induces a type-specific response, it is not known whether primary exposure to all four serotypes simultaneously, as during tetravalent vaccination, skews the immune response toward the production of antibodies with general cross-reactivity, or whether multiple type-specific Ab responses are

simultaneously generated. In this dissertation, we sought to gain insight into the humoral immune response to DENV in the context of the human polyclonal, neutralizing antibody response.

Chapter 3: Context-dependent cleavage of the capsid protein by the West Nile virus protease modulates the efficiency of virus assembly[2]

3.1 Summary

Molecular mechanisms that define the specificity of flavivirus RNA encapsulation are poorly understood. Virions composed of the structural proteins of one flavivirus and the genomic RNA of a heterologous strain can be assembled and have been developed as live-attenuated vaccine candidates for several flaviviruses. In this study, we discovered that not all combinations of flavivirus components are possible. While a West Nile virus (WNV) sub-genomic RNA could readily be packaged by structural proteins of the DENV2 strain 16681, production of infectious virions with DENV2 strain NGC structural proteins was not possible, despite the very high amino acid identity between these viruses. Mutagenesis studies identified a single residue (position 101) of the DENV capsid (C) protein as the determinant for heterologous virus production. C101 is located at the P1' position of the NS2B/3 protease cleavage site at the carboxy-terminus of the C protein. WNV NS2B/3 cleavage of the DENV structural polyprotein was possible when a threonine (Thr101, 16681) but not a serine (Ser101, NGC) occupied the P1' position, a finding not predicted by *in vitro* protease specificity studies. Critically, both serine and threonine were tolerated at the P1' position of WNV capsid. More extensive mutagenesis revealed the importance of flanking residues within the polyprotein in defining the

[2] Adapted from reference 226. **VanBlargan LA, Davis KA, Dowd KA, Akey DL, Smith JL, Pierson TC.** 2015. Context-Dependent Cleavage of the Capsid Protein by the West Nile Virus Protease Modulates the Efficiency of Virus Assembly. J Virol **89:**8632-8642.

cleavage specificity of the WNV protease. A more detailed understanding of the context-dependence of viral protease specificity may aid the development of new protease inhibitors and provide insight into associated patterns of drug resistance.

3.2 Introduction

West Nile virus (WNV) and the four serotypes of dengue virus (DENV1-4) are mosquito-borne viruses of the *Flavivirus* genus that significantly impact public health (227, 228). Despite a clear need, neither vaccines nor therapeutics for WNV or DENV have been licensed for use in humans. The flavivirus genome is an ~11 kb, single-stranded, positive-sense RNA that encodes a single open reading frame flanked by 5' and 3' untranslated regions. The viral genome is translated on endoplasmic reticulum (ER)-derived membranes into a single polyprotein that undergoes co- and post-translational cleavage by the viral protease NS2B/3 and host proteases into ten functionally distinct proteins, including the structural proteins capsid (C), premembrane (prM), and envelope (E) that form the virus particle. During assembly, membrane-anchored prM and E glycoproteins are incorporated into virions as they bud into the ER lumen. The C protein associates with the viral genome in the cytoplasm to form an unstructured nucleocapsid that is incorporated into the budding particle via unknown mechanisms (40). The carboxy-terminus (C-terminus) of the C protein includes a signal sequence, flanked by protease cleavage sites, that directs the translocation of prM into the ER lumen and tethers C to the cytosolic face of the ER membrane. Cleavage at both sites is essential for virion morphogenesis and occurs in a sequential manner (229-231). NS2B/3 first cleaves the C protein on the cytosolic side, resulting in its release from the membrane and increased

exposure of the cleavage site on the luminal side of the ER membrane that is recognized by a cellular signalase. Signalase cleavage remains inefficient until NS2B/3-mediated cleavage has occurred, and uncoupling the coordinated processing of the C protein decreases the nucleocapsid incorporation into virions (232, 233).

The flavivirus protease is a complex of the viral proteins NS2B and NS3, the latter of which contains the proteolytic domain (234-236). NS2B is an essential co-factor that aids in NS3 folding and substrate recognition and contains a hydrophobic domain that tethers the protease to the ER membrane (237, 238). NS2B/3 cleaves at least five positions within the membrane-associated viral polyprotein in addition to the C protein (234, 239, 240). Due to its essential role in flavivirus replication, the proteolytic activity of NS2B/3 is an attractive target for the development of antiviral drugs (reviewed by (241)). The feasibility of designing flavivirus protease inhibitors is suggested by the successful development of protease inhibitors for hepatitis C virus (HCV), a related hepacivirus in the *Flaviviridae* family. Numerous HCV protease inhibitors have been approved for treatment of HCV by the FDA (reviewed in (242)). However, while compounds with inhibitory activity against recombinant flavivirus proteases with activity in cell culture have been described (241, 243-248), no compounds with *in vivo* activity have been described.

The design of peptidic or peptidomimetic inhibitors that bind the active site of viral proteases is informed by a detailed understanding of the specificity of substrate recognition. Alignments of flavivirus NS2B/3 cleavage sites reveal a highly conserved

dibasic motif at the P1 and P2 positions (upstream of the cleavage site), and a small side chain amino acid (G, A, S, or T) at the P1' position (downstream of the cleavage site), according to Schechter and Berger nomenclature (249). This is reflected in the results of substrate specificity profiling studies performed with recombinant proteases of several flaviviruses, including WNV and DENV (250-258). However, while considerable insight has been gained from studies with recombinant proteases or model substrates, it is not known how conditions used in these assays influence cleavage specificity, or if they recapitulate the activity of NS2B/3 in infected cells.

In this study, we have identified novel features of WNV protease substrate-specificity using a cell culture-based assay. In efforts to produce virus particles by *trans* complementation, we discovered that the packaging of a WNV sub-genomic RNA by DENV structural proteins was strikingly strain-dependent. This phenotype was mapped to the substrate specificity of the WNV NS2B/3 protease. WNV NS2B/3 cleavage of the DENV2 C protein was substantially reduced by conservative amino acid substitutions at the P1' position of the cleavage site; cleavage was possible with a glycine or serine, but not threonine at this site. In contrast, cleavage of the WNV C protein was efficient with any of the three residues at the P1' position. This fine specificity was not predicted by studies with recombinant NS2B/3 protease and short peptide substrates (258). We found that the sensitivity of WNV NS2B/3 to changes at the P1' position could be altered when residues at the P6-P2 and P2'-P4' positions were mutated in varying combinations. These findings suggest the context in which the cleavage site is presented has a marked impact

on protease recognition and has implications for studies of flavivirus protease specificity and inhibition.

3.3 Materials and Method

3.3.1 Cell lines.

All cell lines were maintained at 37ºC and 7% CO_2. HEK-293T cells were passaged in Dulbecco's modified Eagle medium (DMEM) containing Glutamax (Invitrogen, Carlsbad, CA), supplemented with 7% fetal bovine serum (FBS) (HyClone, Logan, UT) and 100U/mL penicillin-streptomycin (PS) (Invitrogen, Carlsbad, CA). Raji cells expressing the C-type lectin DC-SIGNR were passaged in RPMI-1640 medium containing Glutamax (Invitrogen, Carlsbad, CA), supplemented with 7% FBS and 100U/mL PS.

3.3.2 Plasmids.

Plasmids encoding sub-genomic replicons of the WNV lineage II strain 956 (herein referred to as WNVrep) and DENV2 strain 16681 (DENVrep) have been described previously (259, 260); both were engineered to express a cassette encoding GFP and a gene conferring resistance to Zeocin. Plasmids that express the CprME structural genes of WNV strain NY99, DENV1 strain Western Pacific-74 (WP), and DENV2 strain 16681 have been described previously (259, 260). Additionally, the CprME structural genes of DENV2 strains New Guinea C (NGC) (GenBank AAA42941) and IQT2913 (GenBank AF100468) were cloned into the expression vector pcDNA6.2 (Invitrogen, Carlsbad,

CA). Single amino acid mutations were introduced into CprME expression constructs using the Quikchange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. Overlap extension polymerase chain reaction (PCR) was used to introduce larger numbers of amino acids changes into the NGC CprME C gene, to construct DENV2 NGC/16681 CprME chimeras, and to add a V5 tag (GKPIPNPLLGLDST) to the amino-terminus of the C protein in NGC and WNV CprME plasmids. All plasmid propagation and cloning procedures were performed using Stbl2 bacteria grown at 30°C (Invitrogen, Carlsbad, CA).

3.3.3 Production of RVPs.

Reporter virus particles (RVPs) were produced by the genetic complementation of a DNA-launched, sub-genomic replicon with a CprME expression plasmid, as described previously (259, 261). Briefly, pre-plated HEK-293T cells were co-transfected with replicon and CprME expression plasmids in a 1:3 ratio by mass using Lipofectamine LTX or Lipofectamine 3000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. Transfected cells were incubated in a low glucose formulation of DMEM containing 25 mM HEPES (Invitrogen, Carlsbad, CA), 7% FBS, and 100U/ml PS. Transfected cells were incubated at 30°C and supernatant was harvested at various times post-transfection, filtered using a 0.22 μm syringe filter, and stored at -80°C.

3.3.4 Measuring the infectious titer of RVPs.

The infectious titer of RVPs was assayed using Raji cells expressing DC-SIGNR, as described previously (259, 261). Briefly, serial two-fold dilutions of RVP-containing supernatant were added to cells, followed by incubation at 37°C for two (WNVrep) or three (DENVrep) days. Infection was scored by flow cytometry as the percent of cells expressing GFP. To calculate infectious titers, only values on the linear portion of virus infectivity titrations were used to solve the following equation: infectious units (IU)/sample volume = (percent GFP positive cells) x (number of cells) x (dilution factor).

3.3.5 Quantification of viral RNA.

The amount of viral RNA in RVP-containing supernatants was measured by quantitative reverse transcription PCR (qRT-PCR). Supernatant was treated with recombinant DNase I (Roche Diagnostics, Indianapolis, IN), followed by lysis and RNA isolation (EZ1 Virus Mini Kit v2.0, Qiagen, Valencia, CA). RNA content was quantified with the Superscript III One-step RT-PCR System with Platinum *Taq* DNA Polymerase (Invitrogen, Carlsbad, CA) and primers specific for the 3' untranslated region of the WNV lineage II replicon (262).

3.3.6 Measuring the efficiency of capsid cleavage.

To measure the efficiency of C protein cleavage by the WNV or DENV protease, V5-CprME expression constructs were transfected into HEK-293T cells as described above. Two days post-transfection, cells were disrupted using RIPA lysis and extraction buffer

(Thermo Scientific, Waltham, MA) supplemented with protease inhibitors (cOmplete mini Protease Inhibitor Cocktail Tablets; Roche Diagnostics, Indianapolis, IN) according to the manufacturers' instructions. Total protein concentration in cell lysates was measured using the Thermo Scientific Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA) and used to normalize loading of samples for analysis by SDS-PAGE and Western blotting. Immunoblots were performed using an anti-V5 mouse monoclonal antibody (Invitrogen, Carlsbad, CA) at 1μg/mL, followed by IRDye 800CW Goat anti-Mouse IgG (1/2500 dilution) (LiCor, Lincoln, Nebraska). Blots were imaged using a LiCor Odyssey infrared imaging system (LiCor, Lincoln, Nebraska). C protein cleavage was detected by the presence of a band corresponding to the V5-tagged C protein (~13 kDa), while uncleaved C protein was detected by the presence of a band corresponding to CprM (~33 kDa). Cleavage efficiency was quantified by comparing the amount of cleaved C (intensity of 13 kDa band) to total C (13 kDa band + 33 kDa band intensities). Cleavage efficiency is presented as the percentage of cleaved over total C protein, plus or minus one standard error of the mean.

3.3.7 Statistical analyses.

Statistical analyses were performed using Prism software version 6.0f for Mac OS X (GraphPad Software, San Diego, CA). Log_{10} IU/mL infectious titer values and cleavage efficiency values were compared by one-way ANOVA followed by Tukey's multiple comparisons test.

3.3.8 Surface electrostatic potential calculation.

Surface electrostatic potential calculations were done with APBS (263) and pdb2pqr (264, 265) using default parameters (protein dielectric = 2.0, solvent dielectric = 78.0). Images were rendered in PyMOL (266).

3.4 Results

3.4.1 DENV2 strain-dependent complementation of a WNV sub-genomic replicon.

Pioneering studies by Alexander Khromykh and colleagues demonstrated that sub-genomic flavivirus replicon RNAs lacking the structural genes could be encapsidated by CprME proteins expressed in *trans* (267). This genetic complementation results in the production of pseudo-infectious virus-like particles capable of only a single round of infection, referred to herein as reporter virus particles (RVPs). RVPs have been used extensively to study flavivirus biology (268-273) and virus-antibody interactions (32, 130, 260, 261, 274), and as tools to screen antiviral compounds (244, 275, 276). The mechanism of flavivirus RNA packaging is not well understood and appears to be relatively non-selective. Replicon RNAs of one flavivirus can be packaged by structural genes derived from another flavivirus (31, 259, 277, 278). Additionally, several flavivirus vaccine candidates are chimeric viruses in which the structural proteins of one virus are inserted into the genomic background of a heterologous virus type (reviewed by (279)). To study mechanisms of flavivirus neutralization, our laboratory has produced RVPs by complementation of a WNV replicon (WNVrep) with structural genes derived from WNV, DENV, and the tick-borne flavivirus Langat (259, 260, 269). In particular, high

titer RVP stocks have been generated by complementation of WNVrep with the structural genes of DENV2 strain 16681 (33, 259).

To expand the DENV2 strains available for study using this approach, we generated CprME structural gene constructs for the strains New Guinea C (NGC) and IQT2913. DENV2 NGC is the strain from which the DENV2 component of the NIAID tetravalent vaccine candidate was derived (15). IQT2913 is an isolate from Iquitos, Peru (280). **Figure 3.1A** shows RVP titers generated by complementation of the WNV replicon with CprME from DENV2 NGC and IQT2913, as well as from flaviviruses previously shown to result in infectious RVPs (DENV2 16681, DENV1 Western Pacific-74 (WP) strain, and the NY99 strain of WNV). Complementation with CprME from WNV, DENV1, and DENV2 16681 produced comparable RVP titers that peaked ~72-96 hours post-transfection, similar to previous observations (259). Mean titers for WNV, DENV1, and DENV2 16681 RVPs collected at 72 hours post-transfection ranged from $1x10^6$ to $4x10^6$ IU/mL (n=3) (**Figure 3.1B**). Conversely, complementation of the WNV replicon with CprME from DENV2 strains NGC and IQT2913 resulted in no detectable RVP production (n=3) (**Figure 3.1A and 3.1B**). These results were surprising, as DENV2 16681 shares 98% and 97% amino acid identity with strains NGC and IQT2913, differing by 17 and 24 amino acid residues within CprME, respectively. Analysis of the RNA content of DENV2 NGC and IQT2913 RVP preparations revealed >1,000-fold lower RNA content than DENV2 16681 RVP-containing supernatants produced in parallel (n=3) (**Figure 3.1C**). Thus, the inability to complement the WNV replicon with structural

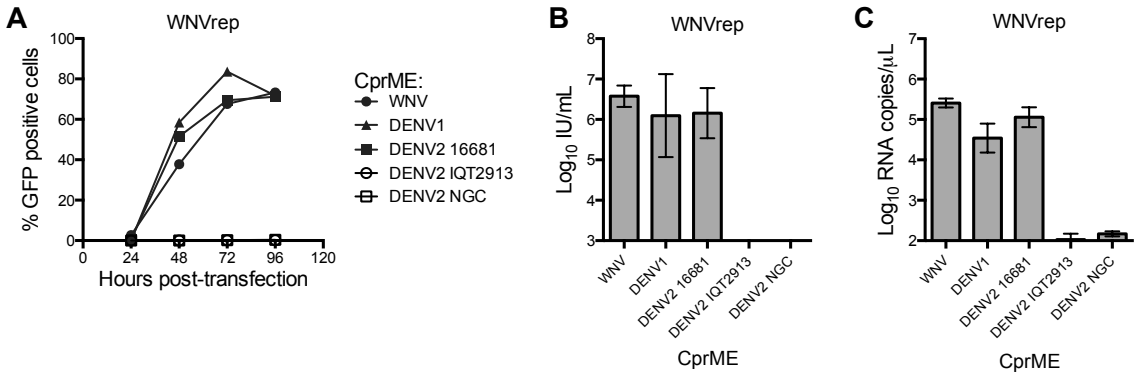


Figure 3.1 DENV2 strain-dependent complementation of a WNV sub-genomic replicon. (**A-C**) RVPs were produced by complementation of a GFP-expressing WNV replicon (WNVrep) and a CprME plasmid encoding the structural genes of WNV (NY99), DENV1 (WP), or DENV2 (strains 16681, NGC, and IQT2913). (**A**) RVPs were harvested at the times indicated post-transfection and used to infect Raji DC-SIGNR cells. Infection was scored by GFP expression two days post-infection using flow cytometry. Error bars represent the range of duplicate infections. The results shown are representative of three independent experiments. (**B**) The infectious titer of RVPs collected 72hr post-transfection was determined. Error bars represent the standard error of the mean for three independent experiments. (**C**) RVPs collected 72hr post-transfection were analyzed for viral RNA content by qRT-PCR. Error bars represent the standard error of the mean for three independent experiments

genes from DENV2 strains NGC or IQT2913 reflected a defect prior to the release of virus particles into the supernatant.

We next performed complementation experiments with a DENV2 sub-genomic replicon of similar design (DENVrep). RVPs produced using this replicon resulted in comparable titers for all three DENV2 strains (16681, NGC, and IQT2913), with mean titers ranging from $1x10^5$ to $5x10^5$ IU/mL at 120 hours post-transfection (n=3) (**Figure 3.2A and 3.2B**). As noted previously (259), RVP production using the DENV replicon was delayed (**Figure 3.1A versus 3.2A**) and less efficient (**Figure 3.1B versus 3.2B**) as compared to experiments with WNVrep.

3.4.2 The C protein governs DENV2 RVP production following WNV replicon complementation.

To determine the molecular basis of the DENV2 strain-specific RVP production, we created chimeras of DENV2 16681 and DENV2 NGC structural proteins (**Figure 3.3A**). Each chimera was evaluated in complementation experiments with the WNV replicon. RVP production studies revealed that all CprME chimeras containing the C gene of 16681, regardless of the origin of the prM or E genes, resulted in robust RVP titers (mean titers $>10^5$ IU/mL, n=3). In contrast, all CprME chimeras encoding the NGC C gene resulted in undetectable RVP production (n=3) (**Figure 3.3B**). These data reveal a critical role for the C protein in modulating DENV2 RVP production using a heterologous viral RNA.

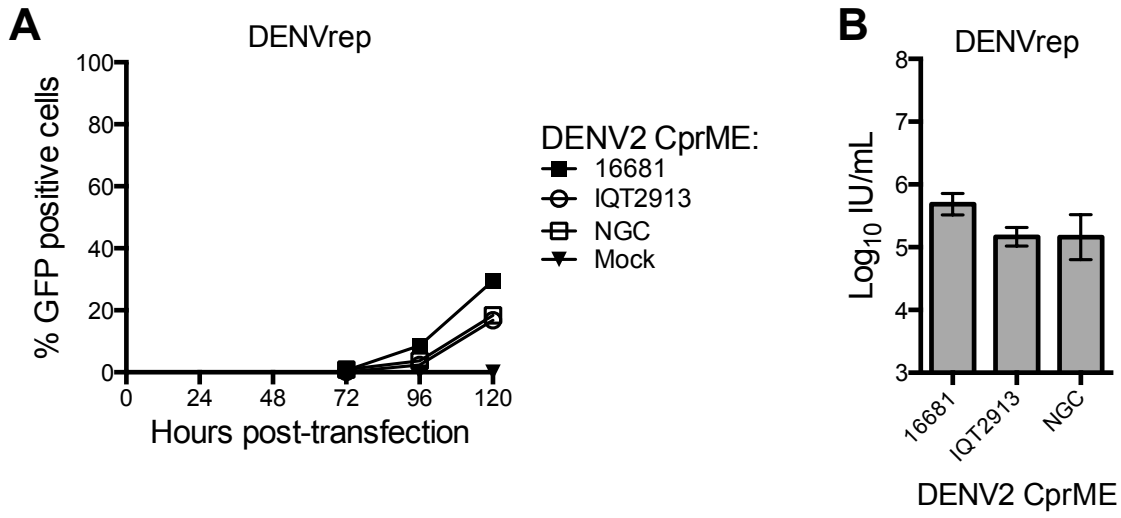


Figure 3.2 DENV2 complementation of a DENV sub-genomic replicon. (**A and B**) RVPs were produced by complementation of a GFP-expressing DENV replicon (DENVrep) with DENV2 CprME (strains 16681, NGC, and IQT2913). (**A**) RVP samples collected at the indicated times post-transfection were analyzed for infectivity of Raji DC-SIGNR cells. Error bars represent the range of duplicate infections. The results shown are representative of three independent experiments. (**B**) The infectious titer of RVPs collected at 120hr post-transfection was determined. Error bars represent the standard error of the mean for three independent experiments.

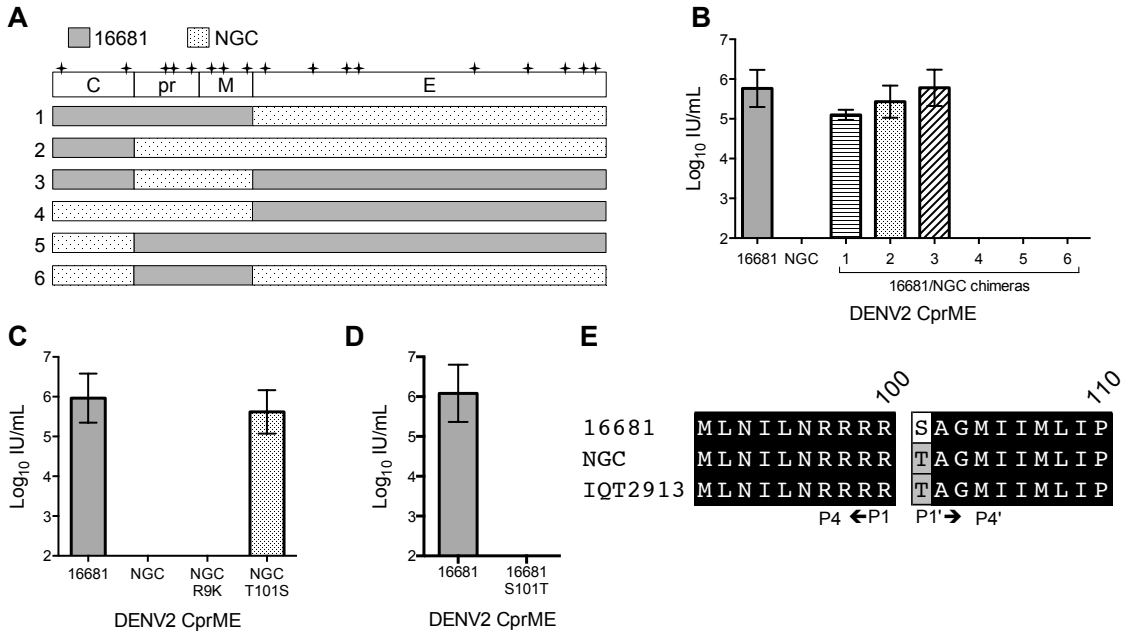


Figure 3.3 Genetic basis of DENV2 strain-dependent complementation of the WNV sub-genomic replicon. (A) Schematics of DENV2 16681/NGC CprME chimeras, with genes from 16681 in grey and genes from NGC in black and white pattern. Locations of amino acid sequence variation between the two strains are indicated by "+" marks. (**B-D**) RVPs were produced by WNVrep complementation with the DENV2 CprME construct indicated on the x-axis. Infectious RVP titer was determined at 72hr post-transfection; error bars represent the standard error of the mean for three independent experiments. Infectious RVP titers are shown for (**B**) DENV2 16681, NGC, and the 16681/NGC CprME chimeras shown in panel A, (**C**) DENV2 16681, NGC, and NGC containing point mutations R9K or T101S in the C protein, and (**D**) DENV2 16681 and 16681 C protein mutant S101T. (**E**) An alignment of sequences surrounding the NS2B/3 cleavage site in the C protein for DENV2 strains 16681, NGC, and IQT2913.

The C proteins of DENV2 16681 and NGC differ at only two amino acid residues (residues 9 and 101). We created NGC variants encoding a single substitution at these positions to correspond to the amino acid found in 16681, and evaluated them in RVP production experiments with WNVrep. Complementation with wild-type (WT) NGC and the NGC R9K variant resulted in undetectable RVP titers (n=3). In contrast, the T101S mutation in the NGC background resulted in RVP production similar to titers obtained with 16681 (>10^5 IU/mL, p=0.36, n=3) (**Figure 3.3C**). A similar pattern was observed following the introduction of mutation T101S into the DENV2 IQT2913 C protein (data not shown). Conversely, the reciprocal S101T mutation in the 16681 C protein rendered this construct incapable of RVP production following complementation of the WNV replicon (**Figure 3.3D**). Together, these results identify C protein residue 101 as the determinant of compatibility between the structural genes of DENV2 and a WNV replicon RNA during virus particle production.

3.4.3 WNV NS2B/3 cannot cleave the DENV2 C protein when a threonine occupies the P1' position.

During polyprotein processing, NS2B/3 cleaves the C protein immediately after residue 100, removing the C-terminal signal sequence and liberating it from its membrane anchor. C protein residue 101 identified above is located immediately downstream of the scissile bond in the P1' position of the cleavage site (**Figure 3.3E**), and therefore has the potential to impact cleavage by the WNV protease. To investigate this hypothesis, we developed an assay to measure C protein cleavage in cells harboring a replicating flavivirus RNA. A V5 tag was inserted at the amino-terminus of the C protein in DENV2

NGC CprME expression constructs (V5-CprME), enabling quantitation of NS2B/3 cleavage efficiency in RVP-producing cells by Western blot. Using this system, capsid cleavage resulted in the production of a V5-tagged C protein that is considerably smaller than its uncleaved C-prM precursor (13 kDa and 33 kDa, respectively). Cleavage studies in HEK293T cells co-transfected with the WNV replicon and WT NGC CprME genes resulted in no detectable cleaved C protein in cell lysates. In contrast, partial cleavage of NGC T101S (the 16681 P1' residue) and NGC T101G (the WNV P1' residue) was observed in experiments performed in parallel (31±16% and 63±12%, respectively; n=4) (**Figure 3.4A and 3.4B**). These results correlated well with the ability of these structural gene constructs to produce infectious RVPs by complementation (**Figure 3.4C** versus **Figure 3.1B and 3.2B**). RVP production with the WT NGC V5-CprME construct resulted in undetectable titers, whereas NGC constructs with T101S or T101G mutations at the P1' site enabled efficient RVP release (>10^5 IU/mL, n=2). By comparison, cleavage experiments using the DENV replicon resulted in efficient cleavage of the C protein regardless of whether the amino acid at residue 101 was a T, S, or G (>90% cleaved C, n=4) (**Figure 3.4A and 3.4B**). RVP titers in the corresponding supernatants were similar for all three structural gene constructs (~3x10^3 IU/mL, n=2). These results demonstrated that even chemically conservative amino acid variation at the P1' position modulates cleavage of the DENV C protein and RVP production in cells replicating a WNV replicon.

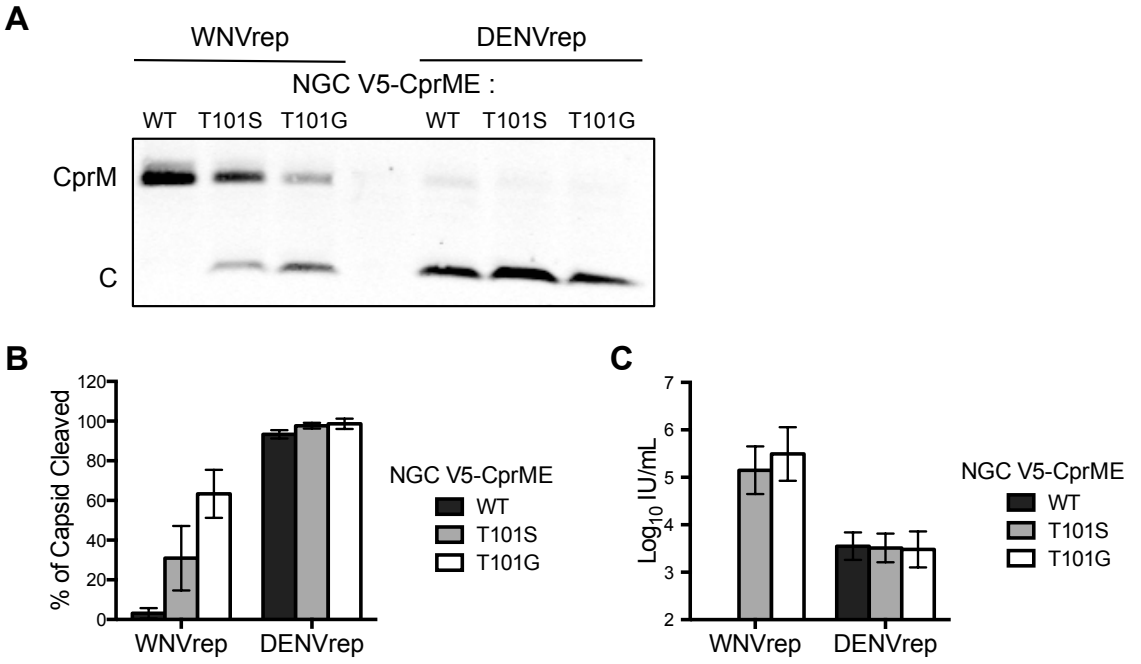


Figure 3.4 Cleavage of the C protein of DENV2 NGC T101 mutants by WNV and DENV NS2B/3. An amino-terminal V5 tag was added to the C proteins of WT NGC, NGC T101S, and NGC T101G CprME constructs. The V5 tagged CprME constructs were used in complementation experiments with WNVrep and DENVrep. Cell lysates were harvested at 48hr post-transfection and analyzed by SDS-PAGE and Western blotting with an anti-V5 mAb. (**A**) A representative blot is shown. The lower band corresponds to cleaved C (~13 kDa), while the upper band corresponds to uncleaved CprM (~33 kDa). (**B**) Mean cleavage efficiency of the C protein from four independent experiments; error bars represent the standard error of the mean. (**C**) Mean infectious titer of RVPs harvested prior to cell lysate collection (48hr post-transfection). Error bars represent the range from two independent experiments.

3.4.4 WNV NS2B/3 efficiently cleaves the WNV C protein when a threonine occupies the P1' position.

Because a T residue in the P1' position of the DENV2 NGC C protein cleavage site prevents processing by the WNV NS2B/3 protease, we hypothesized that introducing a T into the P1' position (residue 106) of the WNV C protein cleavage site would have a similar phenotype. Surprisingly, complementation of the WNV replicon with a WNV G106T variant resulted in efficient RVP production ($3x10^6$ IU/mL, n=3) (**Figure 3.5A**), with only a 0.5 log reduction in titer compared to WT WNV ($p=0.015$, n=3). Likewise, a WNV G106S variant also supported RVP titers similar to WT WNV ($p=0.18$, n=3). Biochemical studies revealed that WNV protease cleavage efficiency of all three constructs was similar (**Figure 3.5B and 3.5C,** n=3, $p>0.05$ for all comparisons). Altogether, these data demonstrate that a threonine in the P1' position of the C protein cleavage site impacts WNV NS2B/3 protease efficiency in a strikingly context-dependent manner.

3.4.5 The specificity of WNV NS2B/3 at the P1' position is influenced by surrounding residues.

We next investigated how the sequence surrounding the protease cleavage site affected WNV protease specificity at the P1' position. We substituted residues adjacent to the incompatible threonine at the P1' position of NGC capsid to those corresponding to the WNV capsid (**Figure 3.6A**). Cleavage of each NGC C protein variant was measured by Western blot of cells expressing the WNV replicon; the efficiently cleaved NGC T101G variant was used as a positive control in these studies. Efficient cleavage was observed

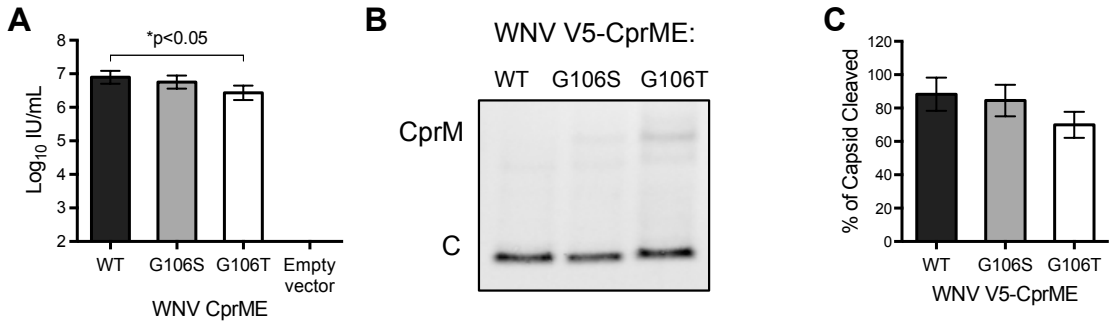


Figure 3.5 Cleavage of the C protein of WNV G106 mutants by WNV NS2B/3. RVPs were produced by complementation of WNVrep with CprME derived from wild-type WNV (WT) or from WNV containing mutations at C protein residue G106 (the P1' position of the WNV C protein cleavage site). (**A**) Shown is the mean infectious RVP titer of supernatant collected 72hr post-transfection; error bars represent the standard error of the mean for three independent experiments. (**B and C**) An amino-terminal V5 tag was added to the C protein in the WNV CprME constructs (WT, G106S, and G106T). Following complementation of WNVrep with the WNV V5-tagged CprME constructs, cell lysates were collected at 48hr post-transfection and analyzed by SDS-PAGE and Western blotting with an anti-V5 mAb. (**B**) A representative blot is shown. The minor mobility shift for the band corresponding to the C protein of G106T was not observed in all independent repeats. (**C**) Mean cleavage efficiency of the WNV C protein; error bars represent the standard error of the mean from three independent experiments.

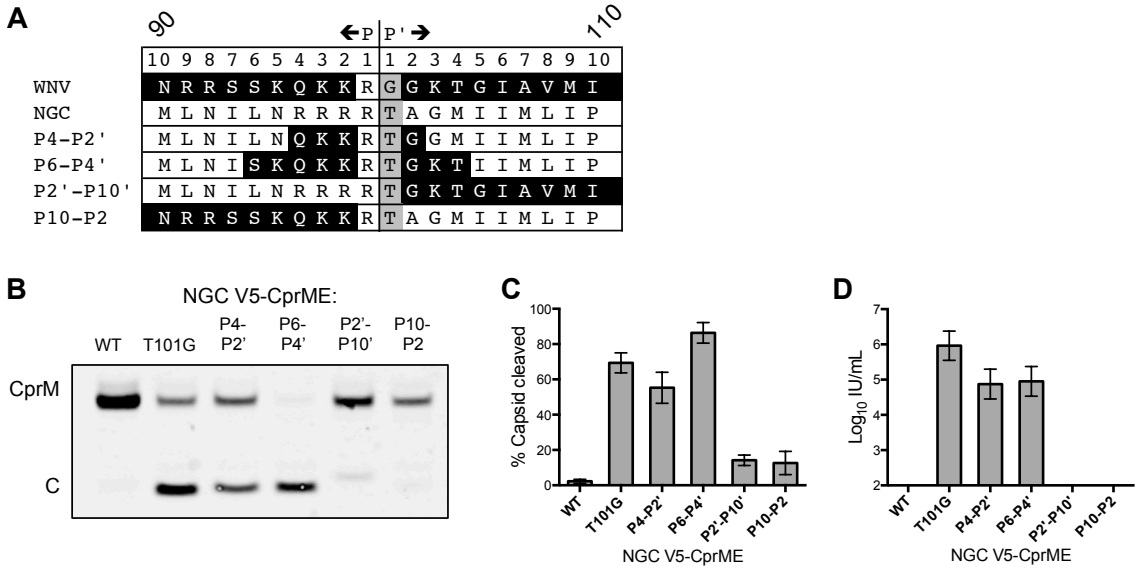


Figure 3.6 Effect of multiple mutations surrounding C protein residue T101 on WNV NS2B/3 cleavage of the NGC C protein. (A) Sequence alignment of the C protein cleavage site of WNV, DENV2 NGC, and DENV2 NGC mutants. Highlighted in gray is the P1' residue; highlighted in black are the substitutions from the WNV cleavage site introduced into each variant. The numbering at the top corresponds to the location in the DENV2 NGC C protein amino acid sequence. (**B-D**) V5-tagged CprME constructs of DENV2 NGC containing a mutation at the indicated C protein cleavage site were used in complementation experiments with WNVrep. Cell lysates were collected and analyzed by SDS-PAGE and subjected to Western blotting with an anti-V5 mAb. For the NGC variants from panel A, shown is a representative blot (**B**), with the mean efficiency of C protein cleavage (**C**) and mean infectious RVP titer (**D**) from four independent experiments; error bars represent the standard error of the mean.

when positions P4-P2' (55±9%) and P6-P4' (86±6%) were mutated (**Figure 3.6B and 3.6C**). Mutations at residues flanking just one side of the cleavage site were poorly cleaved (14±3% and 13±7% for mutants P2'-10' and P10-P2 respectively). Notably, the cleaved C protein of mutant P2'-P10' migrated more slowly during gel electrophoresis than C protein expressed from other NGC constructs (**Figure 3.6B**). In this construct, the order of cleavage may be uncoupled by mutations within the signal sequence, which have the potential to impact the display of the capsid-signal sequence junction and result in a cleaved C protein that retains a C-terminal signal sequence (233). As detailed above, the efficiency of C protein cleavage directly impacted the release of infectious virus particles. The relatively efficiently cleaved capsid variants supported virus production, including NGC T101G ($9x10^5$ IU/mL), mutant P4'-P2' ($7x10^4$ IU/mL; 13-fold reduction from T101G), and mutant P6-P4' ($9x10^4$ IU/mL; 10-fold reduction from T101G), while low-to-undetectable titers for mutants P2'-10' and P10-P2 ($<10^2$ IU/mL) were achieved (**Figure 3.6D**). These results suggest that amino acids directly surrounding the cleavage site influence the ability of WNV NS2B/3 to cleave a substrate when a T occupies the P1' position.

Our data suggested that the ability of WNV NS2B/3 to cleave DENV C proteins with a threonine at the P1' position can be influenced by residues between P4 and P2'. Therefore, we constructed NGC C protein variants with single amino acid substitutions (at the P4, P3, P2, or P2' position) to determine if individual substitutions influence WNV NS2B/3 specificity. Unlike the efficient C protein cleavage detected for NGC mutant P4-P2' encoding four mutations **(Figure 3.6C**), minimal cleavage of variants with

a single mutation at either P4, P3, P2, or P2' was observed (n=3, p>0.25 for WT NGC vs. each mutant) (**Figure 3.7A and 3.7B**). Notably, among these constructs, the C protein of the P2' mutant was most efficiently cleaved (10±3%) (**Figure 3.7B**), and supported RVP production ($5x10^4$ IU/mL; a 51-fold reduction from T101G) (**Figure 3.7C**) to levels similar to that observed when multiple residues were changed (mutants P6-P4' and P4-P2', **Figure 3.6D**).

The difference in cleavage efficiency of the P6-P4' and P4-P2' variants (**Figure 3.6D**) suggests a role for residues farther from the scissile bond studied above. Of interest, the P5 and P3' residues differ in charge between WNV and DENV. We constructed additional NGC C protein variants with single amino acid substitutions at the P6, P5, P3', and P4' positions and evaluated the efficiency of C protein cleavage following complementation with the WNV replicon. Complementation with mutants P6 and P4' resulted in inefficient cleavage (4±1% and 9±5%, respectively) (**Figure 3.7D and 3.7E**) and low but detectable RVP titers ($7x10^3$ IU/mL and $3x10^4$ IU/mL; 59- and 15-fold reductions from T101G titers for mutants P6 and P4', respectively) (**Figure 3.7F**). For mutants P5 and P3', neither cleaved C protein nor infectious RVPs were detectable (**Figure 3.7D-F**).

Altogether, these results indicate that the interactions that mediate WNV NS2B/3 specificity for the P1' site involve multiple surrounding residues. Mutations at the P6, P2', and P4' positions individually had a minor effect on C protein cleavage compared to multiple mutations at the P6-P4' and P4-P2' positions.

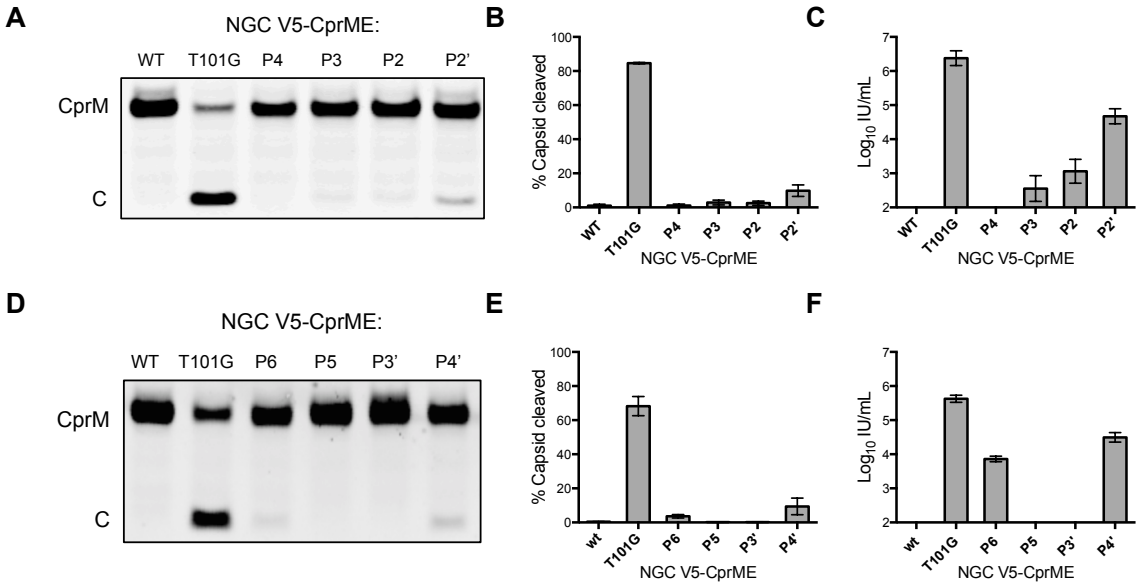


Figure 3.7 Effect of individual mutations surrounding C protein residue T101 on WNV NS2B/3 cleavage of the NGC C protein. V5-tagged CprME constructs of DENV2 NGC containing a mutation at the indicated C protein cleavage site (to match the corresponding residue in the WNV cleavage site) were used in complementation experiments with WNVrep. Cell lysates were collected and analyzed by SDS-PAGE and subjected to Western blotting with an anti-V5 mAb. For mutants P4, P3, P2, and P2', shown is a representative blot (**A**), with the mean efficiency of C protein cleavage (**B**) and mean infectious RVP titer (**C**) from three independent experiments; error bars represent the standard error of the mean. For mutants P6, P5, P3', and P4', shown is a representative blot (**D**), with the mean efficiency of C protein cleavage (**E**) and mean infectious RVP titer (**F**) from three independent experiments; error bars represent the standard error of the mean.

3.5 Discussion

Little is known about the specificity of the flavivirus NS2B/3 protease in the context of viral RNA replication or authentic viral polyprotein substrates (281). Most studies to define protease cleavage site specificity employ short (3-8 amino acids) synthetic peptide substrates and covalently linked and truncated forms of NS2B and NS3 (250-258). Furthermore, these experiments are performed in the absence of membranes, at basic pH (~pH 9), and in the presence of detergents and high concentrations of glycerol. These biochemical approaches have identified a requirement for dibasic residues at P1 and P2 and a small side chain at the P1' position of the cleavage site, though preferences for particular residues within that motif exist and distinct specificities have been noted between viruses. WNV NS2B/3 has been shown to preferentially cleave sites with a K at P2, whereas DENV NS2B/3 most efficiently cleaves sites with an R at this position. In contrast, WNV and DENV NS2B/3 proteases both preferentially cleave sites with an R over a K at the P1 position (250, 258, 282). These studies have indicated that WNV NS2B/3 has a strict preference for G at both the P1' and P2' positions, while DENV NS2B/3 is less selective and can cleave substrates with almost any amino acid at P1' and P2' (258). Another study, however, observed a narrower specificity of the DENV protease at the P1' position (restricted to small, polar amino acids with a strong preference for S) and at the P2' position (a weak preference for acidic residues) (282).

How substrate residues outside of the active site recognition site impact cleavage efficiency has not been well explored for flavivirus proteases. Allosteric and subsite cooperativity have the potential to influence protease substrate specificity (reviewed by

(283)). Allosteric cooperativity occurs when the binding of substrate residues to a protease outside of the active site affects proteolytic efficiency. Subsite cooperativity posits that individual subsite binding may be positively or negatively influenced by binding of surrounding subsites (reviewed in (283)). Both processes have been shown to contribute to HIV-1 protease recognition (284-287). As discussed above, prior studies of the WNV NS2B/3 employed short peptide substrates that do not extend significantly beyond the active site, potentially limiting their utility for identifying all the interactions that impact protease specificity.

To investigate the effect of cleavage site context on WNV NS2B/3 substrate specificity, we studied WNV NS2B/3 processing of DENV and WNV C proteins. WNV NS2B/3 cleaved the DENV2 C protein when a G, but not a T, occupied the P1' position. In contrast, when the P1' residue of the WNV C protein cleavage site was mutated from a G to a T, we observed efficient WNV NS2B/3 cleavage, indicating that a T at P1' is permissive for cleavage only in the context of certain substrates. This context-dependent cleavage specificity could be a result of either allosteric or subsite cooperativity (or both), as the WNV and DENV2 C proteins have sequence differences at residues both within and outside of the protease binding site.

To study the effect of subsite cooperativity on WNV protease specificity, we introduced mutations in a DENV2 CprME construct at residues surrounding the C protein cleavage site. While keeping a T constant at the P1' position (which results in uncleaved C protein in the context of the WT DENV2 NGC sequence), surrounding residues were mutated to

match the WNV C protein cleavage site. We found that WNV NS2B/3 was able to efficiently cleave the DENV2 C protein containing a T at the P1' position when the P4, P3, P2, and P2' positions were mutated simultaneously. When the P6, P5, P3' and P4' positions were additionally mutated, cleavage efficiency was further enhanced. Thus, the sequence context within the active site binding site of the substrate greatly impacts WNV NS2B/3 specificity for the P1' residue. However, when the P6, P5, P4, P3, P2, P2', P3', and P4' mutations were introduced separately, their effect on the efficiency of DENV2 C protein cleavage by the WNV protease was greatly diminished. Thus, the interactions that mediate the context-dependent specificity of WNV NS2B/3 at the P1' position are likely due to the influence of multiple substrate residues.

Notably, one *in vitro* study observed strict specificity of WNV NS2B/3 for a G at P1' (258). This finding is not consistent with the observation that, while most WNV NS2B/3 cleavage sites do contain a G at P1', the P1' position of the cleavage site at the NS3/NS4A junction is occupied by an S. It also contradicts our finding that the WNV and DENV2 C proteins were cleaved by the WNV protease when an S occupied the P1' position. Several differences in experimental design between the studies could account for this discrepancy, including assay conditions (biochemical vs cell-based assay), NS2B/3 source (recombinant, truncated NS2B/3 vs. full-length NS2B/3 from the polyprotein), and the substrate used to probe specificity (9-mer peptides vs. a CprME polyprotein).

Structural basis for WNV NS2B/3 substrate specificity. Efficient peptide cleavage requires accurate placement of the scissile peptide bond in the active site. A view of substrate binding is available in crystal structures for inhibitor-bound WNV NS2B/3 (238, 288, 289) and DENV NS2B/3 (290). The pocket in NS2B/3 occupied by the substrate P1' side chain (the S1' subsite) is lined by the side chains of NS3 residue 36 (DENV I36, WNV A36) and the catalytic histidine (NS3-H51) (238). In structures of both WNV and DENV NS2B/3, the P1' side chain of a protein inhibitor binds identically to the small and relatively hydrophobic S1' subsite (238, 290). In contrast, the S2' subsite for the substrate P2' side chain is a large, open pocket on the enzyme surface between the side chains of NS3 residues 34 and 131. Here the structures differ (NS3 Y34 in WNV *vs*. T34 in DENV; NS3 P131 in WNV *vs*. K131 in DENV). The structures are consistent with the data presented herein that substrate sequences flanking the cleavage site play a role in specificity (290). Our data are also consistent with other observations that NS3-131-132 contributes to specificity of the P2' position, as mutating WNV NS3-P131 and NS3-T132 to match the DENV sequence (P131K+T132P) shifted the specificity of WNV NS2B/3 to that of DENV NS2B/3 (291). Interactions between protease and substrate on the non-prime site of the scissile bond involve the S2 and S3 pockets of the active site, and are occupied by the P2 and P3 positions of the substrate, respectively. Like the situation for the substrate P1' site, the substrate P2 and P3 side chains bind WNV and DENV NS3 identically in the S2 and S3 subsites (238, 289). Substrate-enzyme interactions well beyond the scissile bond and/or cooperativity of subsites are clearly critical.

The subsite cooperativity observed in our study may be a result of restrictions on substrate size by WNV NS2B/3, where the WNV NS2B/3 preference for the smaller substrate residues at P1' (G>T) and P2 (K>R) can be overcome by the introduction of smaller residues at neighboring positions. We have shown that WNV NS2B/3 cleavage of substrates with a T in the P1' position is dependent on the surrounding residues. Notably, when the P4, P3, P2, and P2' positions were mutated to the smaller but chemically similar amino acids found in the WNV C protein cleavage site, WNV NS2B/3 could cleave the DENV2 NGC C protein substrate with a P1' threonine.

Our data together with the available structures show that proper positioning of the peptide substrate is influenced by NS3 interaction with flanking residues at a distance of up to six N-terminal and four C-terminal of the target peptide bond. Although partial activity can be restored with a single substitution at the P1' site, efficient cleavage of natural substrate requires multiple substrate-enzyme interactions. Consistent with our data, WNV and DENV NS2B/3 structures have strikingly different electrostatic surface potential near the active site (**Figure 3.8**). WNV NS2B/3 has a more negatively charged surface in the region surrounding the substrate entrance, while DENV NS2B/3 has a more electropositive surface. The more negative surface of WNV NS2B/3 outside of the active site may account for the increased cleavage efficiency we observed when the P6, P5, P3' and P4' positions were mutated in addition to the P4-P2' positions in NGC mutant P6-P4', as the P5 and P3' positions now contained positively charged lysine residues. While individual mutations at P5 and P3' did not have an effect on WNV NS2B/3 cleavage of

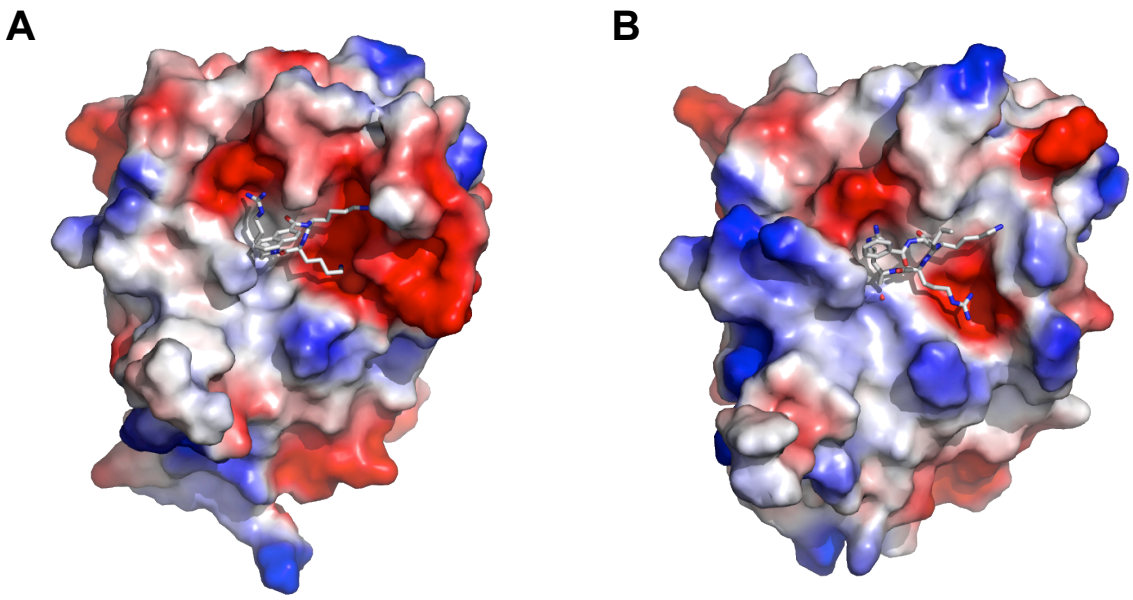


Figure 3.8 Electrostatic surface potential of the WNV and DENV proteases. The surfaces of inhibitor-bound (**A**) WNV NS2B/3 (PDB 3E90) (289) and (**B**) DENV NS2B/3 (PDB 3U1I) (290) are colored according to the electrostatic surface potential from the negative (red -5 kT) to the positive (blue +5 kT).

the NGC C protein, cooperative effects from multiple residues surrounding the cleavage site could mediate cleavage.

Our results indicate that subsite cooperativity involving multiple residues within the P6-P2 and P2'-P4' positions influences the specificity of the WNV protease at the P1' position. This finding has implications for the design of experiments to probe NS2B/3 substrate specificity, as the substrate selected for specificity profiling may drastically affect the results of the screen. Furthermore, molecular details of substrate specificity can be integrated with structural information to facilitate rational inhibitor design.

Chapter 4: The type-specific neutralizing antibody response elicited by a dengue vaccine candidate is focused on two amino acids of the envelope protein[3]

4.1 Summary

Dengue viruses are mosquito-borne flaviviruses that circulate in nature as four distinct serotypes (DENV1-4). These emerging pathogens are responsible for more than 100 million human infections annually. Severe clinical manifestations of disease are predominantly associated with a secondary infection by a heterotypic DENV serotype. The increased risk of severe disease in DENV-sensitized populations significantly complicates vaccine development, as a vaccine must simultaneously confer protection against all four DENV serotypes. Eliciting a protective tetravalent neutralizing antibody response is a major goal of ongoing vaccine development efforts. However, a recent large clinical trial of a candidate live-attenuated DENV vaccine revealed low protective efficacy despite eliciting a neutralizing antibody response, highlighting the need for a better understanding of the humoral immune response against dengue infection. In this study, we sought to identify epitopes recognized by serotype-specific neutralizing antibodies elicited by monovalent DENV1 vaccination. We constructed a panel of over 50 DENV1 structural gene variants containing substitutions at surface-accessible residues of the envelope (E) protein to match the corresponding DENV2 sequence. Amino acids that contribute to recognition by serotype-specific neutralizing antibodies were identified

[3] Adapted from reference 261. **VanBlargan LA, Mukherjee S, Dowd KA, Durbin AP, Whitehead SS, Pierson TC.** 2013. The type-specific neutralizing antibody response elicited by a dengue vaccine candidate is focused on two amino acids of the envelope protein. PLoS Pathog **9:**e1003761.

as DENV mutants with reduced sensitivity to neutralization by DENV1 immune sera, but not cross-reactive neutralizing antibodies elicited by DENV2 vaccination. We identified two mutations (E126K and E157K) that contribute significantly to type-specific recognition by polyclonal DENV1 immune sera. Longitudinal and cross-sectional analysis of sera from 24 participants of a phase I clinical study revealed a markedly reduced capacity to neutralize a E126K/E157K DENV1 variant. Sera from 77% of subjects recognized the E126K/E157K DENV1 variant and DENV2 equivalently (<3-fold difference). These data indicate the type-specific component of the DENV1 neutralizing antibody response to vaccination is strikingly focused on just two amino acids of the E protein. This study provides an important step towards deconvoluting the functional complexity of DENV serology following vaccination.

4.2 Introduction

Dengue virus (DENV) is a mosquito-transmitted flavivirus responsible for 390 million human infections each year (1). Four related serotypes (DENV1-4) circulate in virtually all tropical and sub-tropical regions of the world (2). While DENV infection is often subclinical, clinical symptoms of dengue fever (DF) include a self-limiting febrile illness, myalgia, rash, and retro-orbital pain (3). A more severe clinical illness (dengue shock syndrome/dengue hemorrhagic fever) involving capillary leakage, thrombocytopenia, and hemorrhage has been associated with secondary infections by a heterologous DENV serotype and higher viral loads *in vivo* (86, 292). The incidence of severe DENV disease is rising globally due to increasing co-circulation of multiple DENV serotypes in endemic

areas (2, 8). Currently, there are no specific treatments or approved vaccines for DENV infection.

Flaviviruses encapsidate a single-stranded RNA genome of positive-sense polarity. This ~11kb genomic RNA is translated as a single open reading frame that is cleaved in infected cells by cellular and viral proteases into at least ten proteins (293). The virus encodes three structural proteins (envelope (E), premembrane (prM), and capsid (C)) that associate with a lipid envelope and the viral genome to form the virion (44). Flavivirus assembly occurs on virus-induced membranes derived from the endoplasmic reticulum (ER) (19, 22, 23, 294, 295), resulting in the budding of non-infectious immature virus particles into the lumen. The E protein of immature virions exists as heterotrimeric spikes in complex with the prM protein; sixty of these spikes are organized on the virion with icosahedral symmetry (29, 36, 37). During egress through the secretory pathway, prM is cleaved by a cellular furin-like protease to generate the mature infectious virus particle (26-28). Mature virions are characterized by a dense array of antiparallel E protein dimers orientated roughly parallel to the surface of the virion (39-41). In many cases, this arrangement of E proteins imposes steric constraints for epitope recognition by antibodies (54, 296). The virion maturation process is inefficient for many mosquito-borne flaviviruses, including DENV. Partially mature viruses with structural features of both mature and immature particles may be infectious and differentially interact with antibodies as a function of their prM content (reviewed in (30)).

The humoral response plays an important role in protection against flaviviruses (reviewed in (297)). Development of a neutralizing antibody response is an established correlate of protection following vaccination against yellow fever virus (YFV), Japanese encephalitis virus (JEV), and tick-born encephalitis virus (TBEV) (192-195). Passive transfer of monoclonal antibodies (mAbs) is protective in several animal models of flavivirus infection, including DENV (47, 48, 142, 197-199). The flavivirus E protein is the principal target of neutralizing antibodies (42). Studies with murine and human mAbs have identified neutralizing epitopes on all three structural domains of E (DI-III) (47-56, 296, 298-300). Recent studies with human mAbs suggest the antibody repertoire may differ from that observed in mice (50, 57-60, 301), and have identified a quaternary epitope composed of surfaces on two adjacent E proteins (56-58). Antibodies that bind prM also have been identified frequently in studies of human mAbs and typically possess limited neutralization potential *in vitro* (50, 59-61). Beyond a capacity to directly neutralize the infectivity of virions, antibodies may protect the host via effector functions orchestrated by the constant region of the antibody molecule (136, 302-304).

Development of a DENV vaccine has been a focus of considerable effort for decades. While the dramatic success of other flavivirus vaccines (193, 305, 306) and the pioneering work of Sabin and colleagues (11) suggest effective DENV vaccination is possible, several unique challenges exist. A DENV vaccine must simultaneously protect against four different viruses. In addition, vaccination must not sensitize the recipient to more severe manifestations of disease in the event of breakthrough. The antibody response to DENV results in the production of both type-specific (TS) and cross-reactive

(CR) antibodies that may vary significantly with respect to their capacity to neutralize virus infection (47-49, 215, 216, 307). CR antibodies are hypothesized to contribute to severe clinical outcomes of DENV infection via a process called antibody-dependent enhancement of infection (12). The extensive cross-reactivity of the DENV antibody response complicates serological studies of these viruses and the identification of immune correlates of protection (308, 309). For example, a recent large phase IIb trial of a live-attenuated tetravalent DENV vaccine revealed modest protective efficacy (~30% overall) with no protection at all observed for DENV2 (224). Importantly, despite the absence of protection, vaccine-induced neutralizing antibody was observed for DENV2. Whether this increase in neutralizing titer was associated with a TS- or CR-response is unknown. This trial underscores the importance of understanding the functional complexity of the DENV antibody response.

In this study we sought to identify the immunodominant epitopes recognized by TS-neutralizing antibodies elicited by DENV vaccination of humans. We constructed libraries of DENV1 variants containing substitutions in the E protein at surface exposed residues that differ between the DENV1 and DENV2 components of the NIAID tetravalent vaccine candidate (13). We then screened this library for a reduction in sensitivity to neutralization by sera from DENV1 vaccine recipients, but not DENV2 immune sera from vaccinated subjects. Remarkably, these studies identified two amino acids (E126 and E157) that when mutated significantly reduced the DENV1 TS-neutralizing response of more than 77% of recipients of a monovalent DENV1 vaccine. Our studies, for the first time, identify functionally significant epitopes that comprise a

TS-neutralizing response and provide insight into the complexity of the DENV humoral response.

4.3 Materials and Methods

4.3.1 Cell lines.

All cell lines were maintained at 37°C in the presence of 7% CO_2. HEK-293T cells were passaged in complete Dulbecco's modified Eagle medium (DMEM) containing Glutamax (Invitrogen, Carlsbad, CA), supplemented with 7.5% fetal bovine serum (FBS) (HyClone, Logan, UT) and 100 U/ml penicillin-streptomycin (PS) (Invitrogen, Carlsbad, CA). Raji-DCSIGNR cells were passaged in RPMI-1640 medium containing Glutamax (Invitrogen, Carlsbad, CA), supplemented with 7.5% FBS and 100 U/ml PS.

4.3.2 DENV immune sera and antibodies.

Sera from recipients of phase I studies of candidate monovalent DENV1 or DENV2 vaccines were obtained for study. Initial screening studies were performed using sera pooled from two or three recipients of the DENV1 and DENV2 vaccines, respectively, collected 2-3 years post-vaccination. Neutralizing antibody responses from 24 participants of a DENV1 vaccine study were studied individually (310); sera were collected for study on the indicated day post-vaccination.

4.3.3 Ethics Statement.

Clinical studies were conducted at the Center for Immunization at the Johns Hopkins Bloomberg School of Public Health under an investigational new drug application reviewed by the United States Food and Drug Administration. The clinical protocol and consent form were reviewed and approved by the NIAID Regulatory Compliance and Human Subjects Protection Branch, the NIAID Data Safety Monitoring Board, the Western Institutional Review Board, and the Johns Hopkins University Institutional Biosafety Committee (ClinicalTrials.gov identifiers; NCT00473135, NCT00920517). Written informed consent was obtained from each participant in accordance with the Code of Federal Regulations (21 CFR 50) and International Conference on Harmonisation guidelines for Good Clinical Practice (ICH E6).

4.3.4 Plasmids.

Plasmids encoding a WNV sub-genomic replicon and DENV1 WP CprME structural genes have been described previously (138, 259, 260). An expression construct of the CprME gene of the DENV2 NGC strain was constructed using similar methods and will be described elsewhere (VanBlargan and Pierson, unpublished data). Plasmids encoding structural gene variants with up to three amino acid substitutions were produced by site-directed mutagenesis using the Quikchange Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. All plasmids used in this study were propagated in Stbl2 bacteria grown at 30°C (Invitrogen, Carlsbad, CA).

4.3.5 Selection of DENV1 residues for mutagenesis.

To identify amino acids recognized by TS-neutralizing antibodies in vaccine sera, we targeted residues for mutagenesis that differed between the DENV1 and DENV2 strains used in the NIAID tetravalent vaccine candidate. The envelope proteins of the DENV1 WP strain and the DENV2 NGC strain differ by 158 amino acids. As a metric to narrow our mutagenesis efforts, we focused on residues predicted to be exposed on the surface of the virion. Surface accessibility was estimated using solvent accessible surface areas of the residues determined from the crystal structure of the E protein dimer (PDB ID: 10AN), with a cut-off value of 30 $Å^2$ (UCSF Chimera package) (311, 312). Residues were then further restricted by modeling the dimer onto the mature virion (PDB ID: 1THD) (313). Residues exposed on the surface of the virion were then selected, narrowing the list of candidates to 68 (313). Admittedly, because our selection scheme was based on a static model of the mature virion, this minimalist approach to selecting a core panel of residues had the potential to be complicated by the structural heterogeneity and dynamics of the virus particle. Virion structure is influenced by the maturation state and structural dynamics of the virion (32, 36, 138). Both have the potential to increase the number of residues that may contribute to TS-antibody recognition. Additionally, structural studies of DENV2 at 37°C revealed the virus not only becomes considerably more heterogeneous at this temperature but also appears to adopt a distinct structure(s) at this temperature (41, 314).

4.3.6 Production of DENV RVPs.

DENV RVPs were produced by complementation of a WNV replicon with plasmids encoding the structural genes of DENV as described previously (138, 259). Briefly, pre-plated HEK-293T cells were transfected with plasmids encoding the WNIIrepG/Z replicon and DENV CprME in a 1:3 ratio by mass, using Lipofectamine LTX (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. Four hours post-transfection, culture media were replaced with a low-glucose formulation of DMEM containing 25 mM HEPES (Invitrogen, Carlsbad, CA), 7% FBS, and 100 U/ml PS, and incubated at 30°C. RVP-containing supernatant was collected at multiple time points starting at 72 hours post-transfection, filtered using a 0.22μm syringe filter, and stored at -80°C. The low-glucose DMEM was replenished after each harvest.

To generate more homogenous populations of RVPs with low to undetectable uncleaved prM, complementation experiments were modified to include a plasmid expressing human furin (furin DENV) in a 1:3:1 ratio of replicon, CprME, and furin by mass. In order to generate RVP populations that retained significant levels of uncleaved prM (FI-DENV), medium from transfected cells was exchanged with medium supplemented with 50 μM furin inhibitor (FI) Dec-RVKR-CMK at four hours post-transfection (Enzo Life Sciences, Farmingdale, NY).

The efficiency of prM cleavage in RVP preparations was determined by Western blotting as previously described (32, 315). Briefly, DENV RVPs were partially purified over a 20% sucrose cushion by ultra-centrifugation. Pelleted RVPs were lysed with in buffer

containing 1% Trition, 100 mM Tris, 2 M NaCl, and 100 mM EDTA. The protein content of lysates was analyzed using E- and prM-reactive mAbs (4G2 and GTX128093 (Genetex), respectively) at 1 μg/ml. The efficiency of prM cleavage was evaluated on blots normalized by loading equivalent E protein.

4.3.7 Measuring the infectious titer of DENV RVPs.

The infectious titer of each RVP stock was determined using Raji cells that express the flavivirus attachment factor DCSIGNR as described previously (130, 259, 316). Cells were infected with serial two-fold dilutions of transfection supernatant, incubated at 37°C for two days, and then scored for GFP expression by flow cytometry. Only data from the linear portion of the virus dose-infectivity curves were used to compare RVP titers. Infectious titer was calculated using the formula: IU/sample volume = (percent GFP positive cells) x (number of cells) x (dilution factor).

4.3.8 DENV neutralization assays.

DENV RVP stocks were diluted and incubated with serial dilutions of mAb or serum for one hour at room temperature prior to the addition of Raji-DCSIGNR cells, unless specified otherwise. Infections were carried out at 37°C for 48 hours and infectivity was scored as the fraction of GFP-expressing cells determined using flow cytometry. Antibody-dose response curves were analyzed using non-linear regression analysis (with a variable slope) (Graphpad Software, La Jolla, CA). Data are expressed as the

concentration of antibody (EC50) or serum dilution (NT50) required to reduce infection by half.

4.3.9 Statistical analysis.

Statistical analyses were performed using Prism software (GraphPad). Log EC50 or Log NT50 values were compared using Student's *t*-test when comparing two samples. For comparisons of more than two samples, Log NT50 values were compared by one-way ANOVA followed by Tukey's multiple comparisons test or, where indicated, Šidák correction for multiple comparisons.

4.4 Results

4.4.1 Strategy for identifying epitopes recognized by type-specific antibodies elicited by DENV1 vaccination.

The goal of this study was to identify epitopes recognized by DENV1 TS-neutralizing antibodies elicited by vaccination. Our analyses employed immune sera collected during the clinical evaluation of individual components of the NIAID tetravalent vaccine candidate (reviewed by (13)). The attenuated DENV1 in this vaccine (rDEN1Δ30) is derived from the South Pacific genotype 4 Western Pacific (WP) strain (14). The DENV2 component of the tetravalent formulation (rDEN2/4Δ30(ME)) is the Southeast Asian genotype 2 New Guinea C strain (NGC) (15). The envelope proteins of these viruses differ by 158 amino acids (**Figure 4.1A and 4.1B**); 68 amino acids that differ between these two strains are predicted to be accessible to solvent on the surface of the mature

DENV virion (**Figure 4.1C,** see Materials and Methods) (313). Both rDEN1Δ30 and rDEN2/4Δ30(ME) vaccines were tested as monovalent formulations in phase I clinical studies in humans (317, 318). To identify amino acids recognized by TS-neutralizing antibodies, we created a panel of DENV1 variants to replace surface-accessible residues on the mature DENV1 virion with those found on DENV2 NGC (detailed below). This panel of mutants was then screened using pooled immune sera collected during monovalent DENV1 and DENV2 vaccine studies for those exhibiting reduced sensitivity to neutralization by DENV1, but not DENV2, immune sera.

Construction and functional characterization of libraries of DENV1 E protein variants were performed using DENV reporter virus particles (RVPs). RVPs are produced by complementation of a self-replicating sub-genomic flavivirus replicon with a plasmid encoding viral structural genes. These virus particles are capable of only a single round of infection and allow virus entry to be scored as a function of reporter gene expression. Flavivirus RVPs have been used extensively to study the functional properties of anti-flavivirus antibodies (32, 130, 171, 199, 260, 319). This technology allows for the rapid generation of structural gene variants by exchanging the plasmids used in complementation studies, simplifying the construction and characterization of a large library of DENV1 mutants. Furthermore, because the RVPs are not passaged in cell culture, the genetic stability of a particular mutation does not limit its utility in neutralization studies.

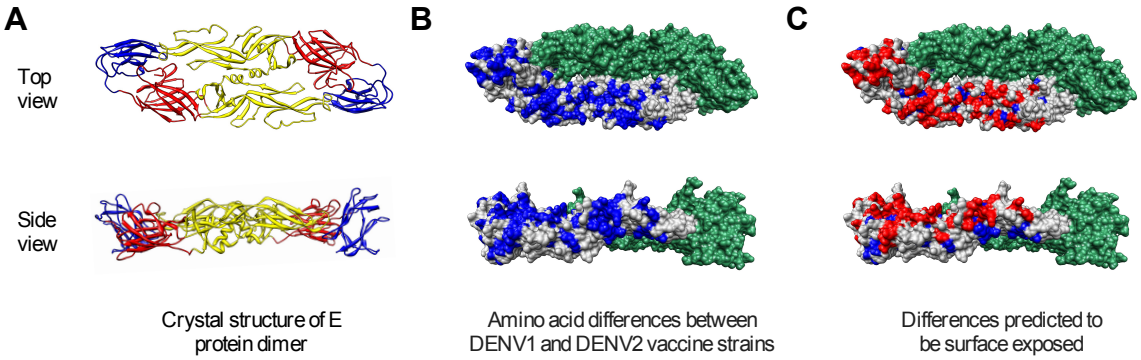


Figure 4.1 Surface-accessible residues that differ between DENV1 and DENV2 identified for mutagenesis. The flavivirus E protein contains three distinct domains (DI-III) and forms antiparallel dimers on the mature virus particle. Shown is the crystal structure of the soluble ectodomain of the DENV2 E protein dimer (PDB 1OAN) as viewed from the top (top panels) and side (bottom panels). (**A**) Ribbon diagram of the E protein with DI, II, and III colored in red, yellow, and blue, respectively (311). (**B**) The 130 amino acid residues in the soluble ectodomain that differ between the DENV1 and DENV2 components of the NIAID candidate tetravalent vaccine are highlighted in blue on one E protein; the second E protein of the dimer is shaded in green. (**C**) Surface accessibility was estimated using solvent accessible surface areas of the residues determined from the crystal structure (UCSF Chimera package), with a cut-off value of 30 $Å^2$ (312). Residues selected for study were restricted to the top of the dimer. The 68 residues identified as surface-accessible differences between DENV1 WP and DENV2 NGC are shown in red.

Immune sera used to characterize and screen DENV1 mutant libraries were pooled from recipients of DENV1 or DENV2 vaccine candidates (two and three vaccine recipients, respectively) (317, 318). The neutralization titer (NT50) of antibodies in these pooled serum samples was determined using wild type (WT) DENV1 WP and DENV2 NGC RVPs. As expected, antibodies present in the DENV1 immune sera efficiently neutralized DENV1 RVPs (Log NT50 2.67±0.04, n=11) (**Figure 4.2A and 4.2B**). Cross-reactive neutralizing antibodies in the sera, measured using DENV2 RVPs (Log NT50 1.32±0.05, n=11), were significantly less potent when compared to DENV1 neutralization (23-fold; $p<0.0001$). A similar pattern of TS- and CR- neutralization was observed in reciprocal studies with DENV2 immune sera (**Figure 4.2C and 4.2D**), which neutralized DENV2 RVPs (Log NT50 3.06±0.03, n=11) at significantly greater dilutions than DENV1 RVPs (Log NT50 1.80±0.04, n=9) (20-fold, $p<0.0001$).

4.4.2 Construction of a library of DENV1 variants with individual substitutions to match DENV2 at surface-accessible differences.

To disrupt TS epitopes recognized by DENV1 immune sera, we created a library of RVPs in which the 68 surface-accessible residues that vary between the DENV1 and DENV2 components of the vaccine (**Figure 4.1**) were used to guide construction of a panel of 54 DENV1 variants containing only one, two, or three substitutions each; in cases where adjacent residues were selected for mutagenesis they were introduced into the same RVP. All 54 DENV1 variants were infectious, albeit to differing degrees (n=2-5) (**Figure 4.3**). The sensitivity of each variant in this panel to neutralization by DENV1

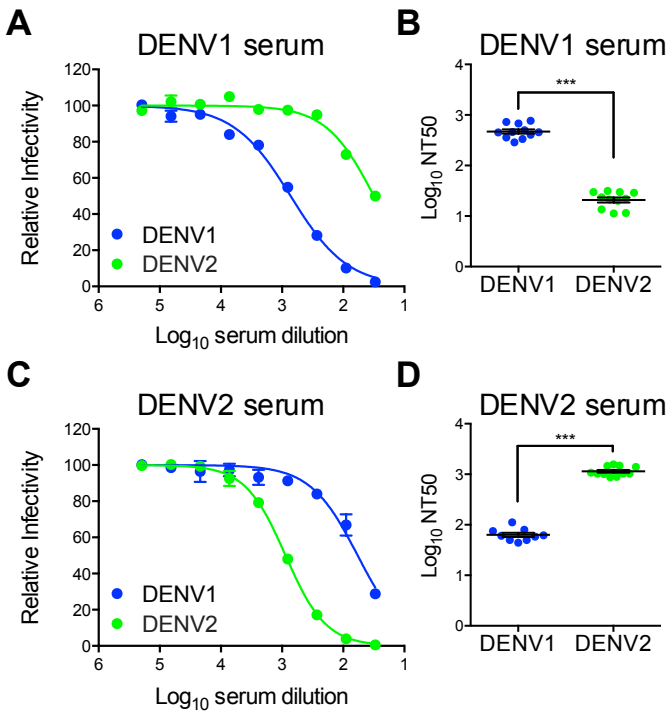


Figure 4.2 Type-specific neutralization by DENV immune sera from a clinical vaccine trial. Pooled immune sera from a DENV1 or DENV2 vaccine study were tested for their capacity to neutralize DENV1 and DENV2 RVPs. Sera pooled from multiple vaccinees (two and three for DENV1 and DENV2, respectively) were used in neutralization experiments by incubating RVPs with serial dilutions of immune sera for one hour at room temperature, before addition to Raji-DCSIGNR cells. After incubation at 37°C for two days, infection was measured using flow cytometry. Dose response curves for (**A**) DENV1 sera and (**C**) DENV2 sera are expressed relative to the infectivity of the RVPs in the absence of serum. The concentration of sera indicated on the x-axis is expressed as Log_{10} (dilution factor of serum). Error bars represent the standard error of duplicate infections. Dose response curves shown in (**A**) and (**C**) are representative of 11 and nine independent experiments, respectively, performed using at least three independent RVP preparations. Neutralization titer (NT50) values were determined by nonlinear regression analysis using Prism software (GraphPad), and are summarized for (**B**) DENV1 sera and (**D**) DENV2 sera. Error bars represent standard error of the mean. ***$p<0.0001$.

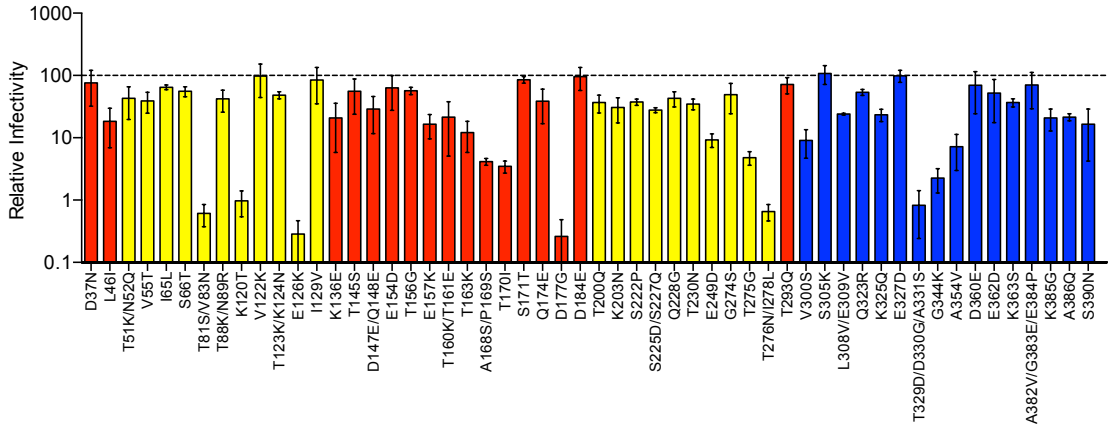


Figure 4.3 Infectivity of DENV1 E protein variants. A panel of 54 DENV1 RVP variants containing single, double, or triple amino acid changes was constructed by site-directed mutagenesis. This panel represented all surface-accessible residues identified in **Figure 4.1C**. The infectious RVP titer for each variant was determined concurrently with WT DENV1 using Raji-DCSIGNR cells. Values are the mean relative titer as compared to WT DENV1 infectivity measured in parallel from at least two independent RVP preparations; error bars represent standard error of the mean. Variants located in domains I, II and III of the E protein are colored in red, yellow, and blue, respectively.

immune sera was tested, and three patterns emerged. The majority of DENV1 variants (81%) were neutralized to the same extent as WT DENV1 tested in pairwise experiments (**Figure 4.4A and D**). Eight variants in this panel (15%) revealed a small (1.5- to 2-fold) but reproducible increase in sensitivity to neutralization (**Figure 4.4B and D**). These variants were also typically more sensitive to neutralization by the DII-fusion loop reactive mAb E60 (**Figure 4.5**). Mechanisms with the potential to increase neutralization sensitivity in this context are discussed below. Finally, two mutants, E126K and E157K, exhibited a statistically significant decrease in sensitivity to neutralization by pooled DENV1 sera (3.5-fold ($n=10$, $p<0.0001$) and 2.4-fold ($n=10$, $p<0.0001$), respectively) (**Figure 4.4C and 4.4D)**. Neither of these two mutations conferred an altered sensitivity to neutralization by mAb E60 (**Figure 4.5**).

To investigate whether a single DENV1 variant encoding both E126K and E157K mutations had an even further reduction in neutralization sensitivity, we combined them into a single construct (**Figure 4.6A**). RVPs produced using this DENV1 E126K/E157K variant were able to infect cells, albeit with greatly reduced titer as compared to WT DENV1 RVPs (>1,000 fold, $p<0.001$ for all time points, $n=2$-4) (**Figure 4.6B**). Neutralization studies using single and double mutants revealed the DENV1 E126K/E157K variant was more resistant to neutralization by DENV1 immune sera than either single mutant (Log NT50 1.79±0.06, $n=11$) ($p<0.0001$ for both comparisons) (**Figure 4.6C and 4.6D**).

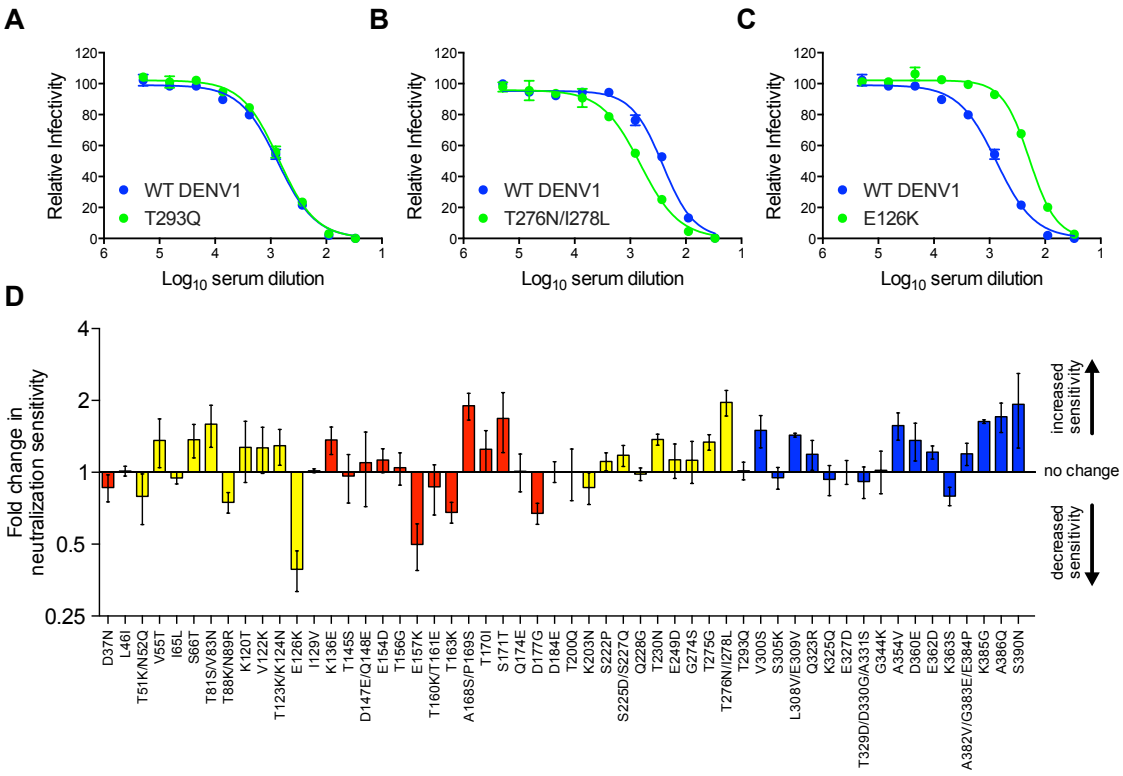


Figure 4.4 Impact of mutations on the neutralization potency of DENV1 immune serum. The sensitivity of the 54 DENV1 variants to neutralization by DENV1 immune serum was compared to WT DENV1. (**A-C**) Examples of the three patterns of neutralization by DENV1 immune sera observed are shown. Error bars represent the standard error of duplicate infections. (**D**) Neutralization sensitivities of all DENV1 variants to DENV1 immune sera are depicted as the mean fold increase in neutralization sensitivity ([NT50 variant]/[NT50 WT]); error bars represent standard error of the mean of 2-5 independent experiments. Variants located in domains I, II and III of the E protein are colored in red, yellow, and blue, respectively.

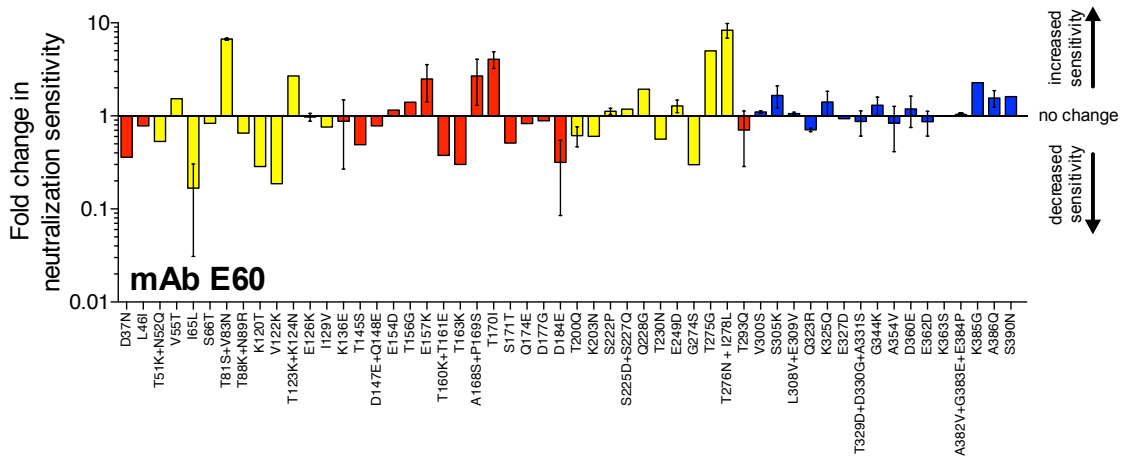


Figure 4.5 Sensitivity of DENV1 variants to neutralization by mAb E60. Each member of the panel of 54 DENV1 variants was tested in parallel with WT DENV1 for sensitivity to neutralization by CR mAb E60. Dose response curves were generated by incubating serial dilutions of antibody with RVPs for one hour at room temperature, before addition of Raji-DCSIGNR cells. EC50 values for each curve were determined by nonlinear regression analysis using Prism software (GraphPad), and are depicted as the fold-change in neutralization sensitivity from WT DENV1 ([EC50 WT]/[EC50 variant]). The experiment was repeated for a subset of the panel to capture the variability of the assay; error bars, when present, represent standard error of the mean from 2-3 independent experiments for 25 of the variants.

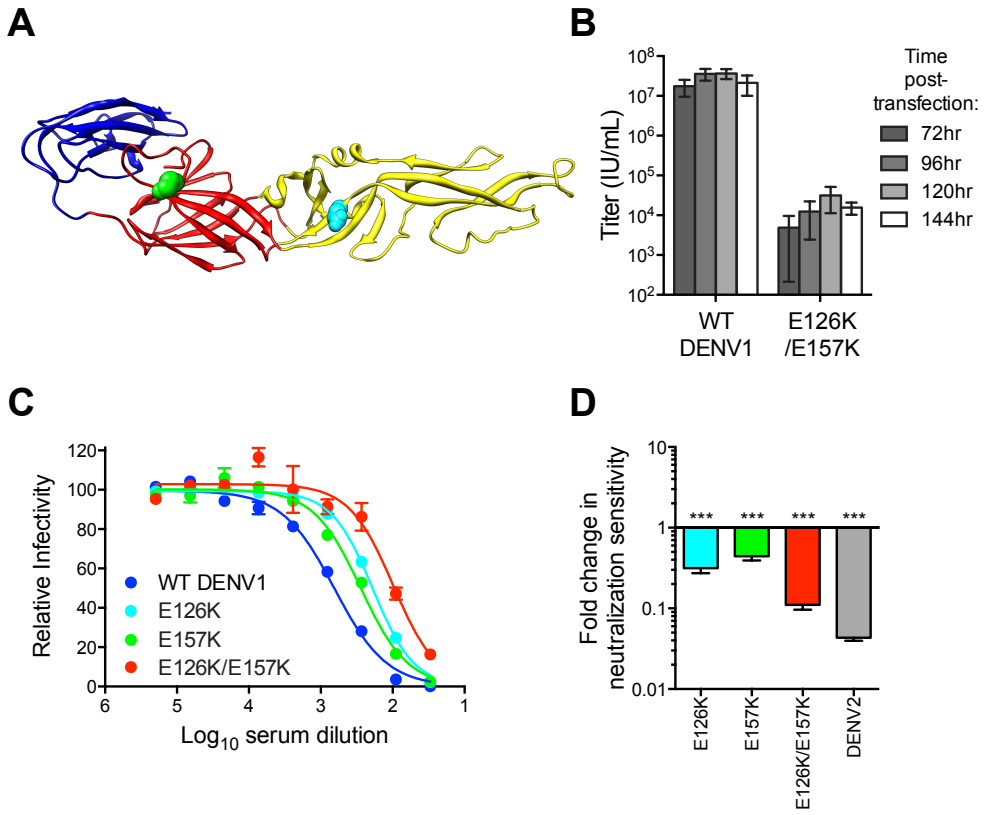


Figure 4.6 Combined effect of DENV1 mutations E126K/E157K on the neutralization potency of DENV1 immune serum. (**A**) The location of residues E126 and E157 are highlighted on the E protein crystal structure as cyan and green spheres, respectively. E protein domains are colored as in **Figure 1**. (**B**) Infectious titer of DENV1 E126K/E157K RVPs harvested at four time points post-transfection was determined in parallel studies with WT DENV1 using Raji-DCSIGNR cells; error bars represent the standard error of the mean of 2-4 independent experiments. (**C and D**) DENV1 E126K/E157K RVPs were tested for sensitivity to neutralization by the DENV1 immune serum. (**C**) Representative dose-response curves for the single and double mutants are shown; error bars represent the standard error of duplicate infections. (**D**) The neutralization sensitivity is summarized as the fold-increase in NT50 from WT DENV1 for the single mutants E126K and E157K (n=10), the E126K/E157K double mutant (n=11), and DENV2 (n=11); error bars represent the standard error of the mean. ***p<0.0001 for a comparison of the Log NT50 values to WT DENV1 by an ANOVA followed by Tukey's multiple comparisons test.

4.4.3 Characterization of the E126K/E157K DENV1 variant.

Our screening data with pooled immune sera suggested antibodies that bound epitopes containing residues E126 and E157 were a functionally significant component of the polyclonal antibody response to DENV1 vaccination. To validate this interpretation, we performed control experiments to address aspects of flavivirus biology with the potential to reduce the apparent sensitivity of flaviviruses to antibody-mediated neutralization.

First, we conducted experiments to demonstrate that the reduced sensitivity of the E126K/E157K variant to neutralization was not an artifact of its low titer and/or an overall disruption of the antigenic surface of the virion. An assumption of quantitative neutralization studies is that the observed neutralizing activity is dependent solely on the concentration of antibody and its affinity/avidity for viral antigens; the results of neutralization studies should be independent of the amount of antigen/virus in the experiment (320, 321). Excess antigen in virus preparations (due to a reduced specific infectivity) may shift antibody dose-response curves towards higher concentrations of antibody if present in sufficient amounts to reduce the concentration of free antibody in solution (260). To rule out the confounding effects of excess antigen in preparations of the E126K/E157K variant, we performed control studies using TS-neutralizing mAbs that have been mapped to the DIII lateral ridge (47). Neutralization by these mAbs should not be affected by the DI/DII mutations. Both mAb DENV1-E103 (**Figure 4.7A**, n=8) and mAb DENV1-E105 (**Figure 4.7B**, n=5) neutralized WT DENV1 and DENV1 E126K/E157K RVPs to an equivalent extent ($p= 0.14$ and 0.57, respectively). Confirmatory studies with six additional DENV1 mAbs yielded similar results; in each

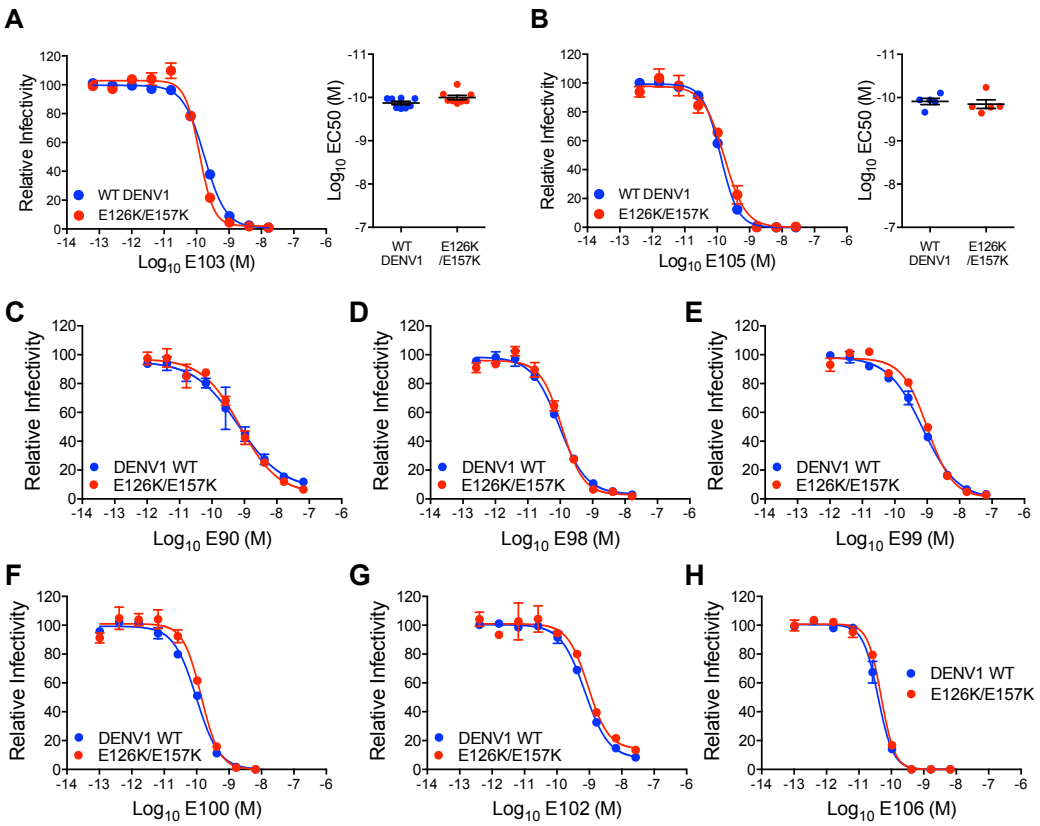
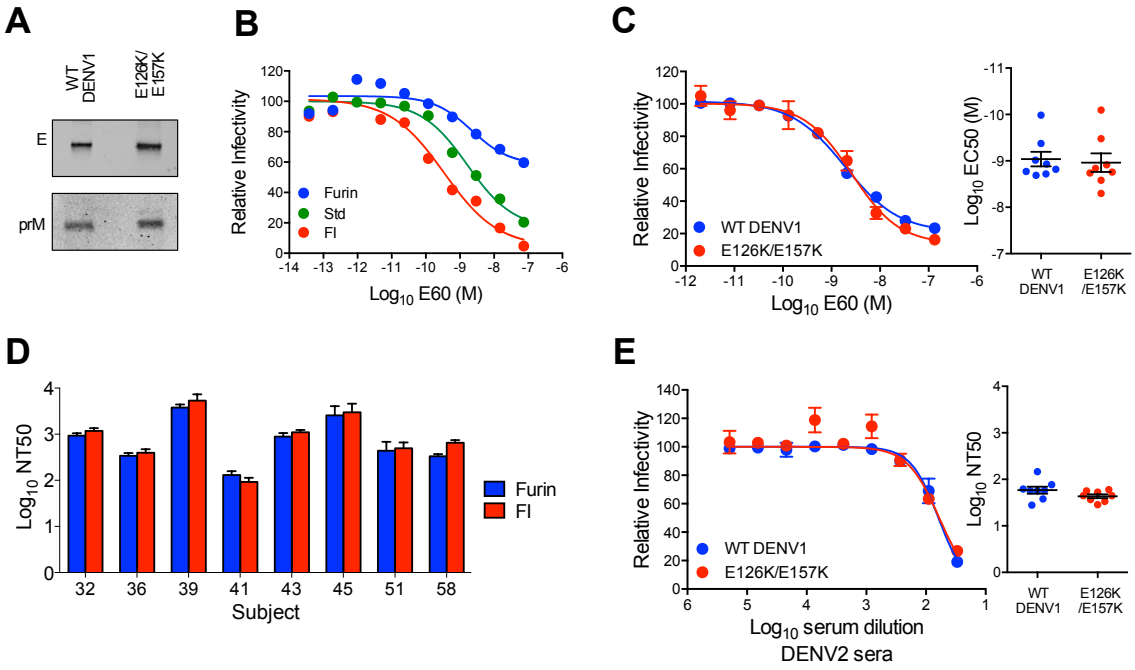


Figure 4.7 Neutralization of DENV1 E126K/E157K by DENV1 mAbs. WT DENV1 and DENV1 E126K/E157K RVPs were evaluated for sensitivity to neutralization by DENV1 DIII-binding mAbs. (**A** and **B**) Within each panel, representative dose response curves for each antibody are shown on the left; error bars represent the standard error of duplicate infections. Plots on the right show the EC50 values obtained from independent experiments; error bars represent standard error of the mean. The antibodies tested were (**A**) mAb E103 (n=8, p=0.14), and (**B**) mAb E105 (n=5, p=0.57). (**C**-**H**) WT DENV1 and DENV1 E126K/E157K RVPs were evaluated for sensitivity to neutralization by six additional DENV1 mAbs that bind diverse epitopes on DIII (47). The mAbs used were (**C**) E90 (N-terminal region and BC-loop); (**D**) E98 (F- and G-strands); (**E**) E99 (A-strand); (**F**) E100 (A-strand, BC and DE loops); (**G**) E102 (N-terminal region and the BC loop); and (**H**) E106 (A-strand, BC, DE, and FG loops). Dose response curves shown are representative of two independent experiments; error bars represent the standard error of duplicate infections. EC50 values for WT and the variant were less than 2-fold different in all cases.

instance we observed less than a two-fold difference in the EC50 of WT and the E126K/E157K variant (**Figure 4.7C-H**, n=2). Altogether, these results demonstrate that studies of the neutralization sensitivity of the DENV1 E126K/E157K variant are not confounded by excess antigen arising from a reduction in the specific infectivity of the virus.

We have shown previously that changes in the efficiency of virion maturation can markedly impact the sensitivity of flaviviruses to neutralization by antibodies (32). Because mutations in the E protein have the potential to impact the efficiency of prM cleavage, we investigated the maturation state of the DENV1 E126K/E157K variant using biochemical and functional approaches. The prM content of the DENV1 E126K/E157K variant was found to be similar to WT by Western blot **(Figure 4.8A)**. The DII-FL-reactive mAb E60 neutralizes flaviviruses in a maturation state-dependent fashion and serves as a sensitive functional probe for changes in virion structure that expose this otherwise cryptic epitope on the mature virion (32, 138). Manipulating the efficiency of prM cleavage modulates the potency of E60 against DENV1 WP; increasing the efficiency of virion maturation reduces sensitivity of the virion to neutralization **(Figure 4.8B)**. That no significant difference in the potency of E60 was observed when studying WT DENV1 (EC50=$1.3x10^{-9}$±$2x10^{-10}$M, n=8) and DENV1 E126K/E157K (EC50=$1.9x10^{-9}$±$5x10^{-10}$M, n=8) (p=0.68) (**Figure 4.8C**) suggests the efficiency of virion maturation of WT and the E126K/E157K variant are similar on infectious viruses. Finally, using RVP populations in which the efficiency of prM cleavage was greatly enhanced (furin RVPs) or reduced via treatment of cells with furin inhibitor (FI RVPs)

Figure 4.8 Characterization of the E126K/E157K DENV1 variant. (**A**) WT DENV1 and E126K/E157K RVPs were analyzed by Western blot with an anti-E mAb and an anti-prM mAb. The efficiency of prM cleavage was evaluated on blots normalized by loading equivalent E protein. (**B**) WT DENV1 RVPs were produced using standard methods (Std), in the presence of high levels of human furin expression (furin), or in cells treated with furin inhibitor (FI) and then tested for sensitivity to neutralization by mAb E60. Three independent experiments were performed; representative dose response curves are shown. Error bars represent the standard error of duplicate infections. (**C**) DENV1 E126K/E157K was evaluated for sensitivity to neutralization by mAb E60 as compared to WT DENV1. Representative dose response curves are shown on the left; error bars represent the standard error of duplicate infections. EC50 values from independent experiments are shown in the right panel; error bars represent standard error of the mean (n=8, p=0.68). (**F**) Furin- and FI-DENV1 RVPs were tested for sensitivity to neutralization by sera from DENV1 vaccine recipients. Error bars represent standard error from three independent experiments. Statistical evaluation using ANOVA followed by a Šidák correction for multiple comparisons failed to identify a difference between Furin- and FI-DENV1 RVPs ($p>0.05$ for each pair). (**G**) DENV1 E126K/E157K was evaluated for sensitivity to neutralization by pooled sera from DENV2 vaccine recipients. A representative dose response curve is shown on the left; error bars represent the standard error of duplicate infections. NT50 values obtained from eight independent experiments are shown on the right; error bars represent standard error of the mean (p=0.08).


A
WT DENV1
E126K/ E157K
E
prM
B
Relative Infectivity
Furin
Std
FI
Log10 E60 (M)
C
Relative Infectivity
WT DENV1
E126K/E157K
Log10 E60 (M)
Log10 EC50 (M)
WT DENV1
E126K /E157K
D
Log10 NT50
Furin
FI
Subject
E
Relative Infectivity
WT DENV1
E126K/E157K
Log10 serum dilution
DENV2 sera
Log10 NT50
WT DENV1
E126K /E157K

and DENV1 immune sera from eight vaccine recipients, we established that the TS-neutralizing activity in these sera was not markedly sensitive to the maturation state of the virus particle (**Figure 4.8D**). In each instance, furin- and FI-RVPs were neutralized equivalently. Altogether our data strongly suggest that the reduction in sensitivity of the DENV1 E126K/E157K variant to neutralization by TS antibodies present in vaccine immune sera is not an artifact of a change in the efficiency of virion maturation.

The accessibility of antibody epitopes on the virion may also be modulated by changes in the structure of the virion as it samples multiple conformations at equilibrium (known as viral "breathing") (138, 173, 175, 322, 323). The impact of viral dynamics on antibody-mediated neutralization is most readily observed with antibodies that bind epitopes predicted to be poorly exposed on the surface of the mature virion. The neutralization potency of E60 is also sensitive to viral structural dynamics (138). That E60 neutralized both WT and the DENV1 E126K/E157K variant with equivalent efficiency (**Figure 4.8C**) indicates that the reduced sensitivity of this variant to neutralization by DENV1 immune sera is not a result of a change in the extent of viral "breathing".

Finally, studies with pooled DENV2 immune sera revealed these mutations only altered sensitivity to TS antibodies and not CR antibodies. The sensitivity of DENV1 E126K/E157K RVPs to neutralization by cross-reactive DENV2 immune sera was similar to WT DENV1 RVPs (1.3-fold, n=8, p=0.08) (**Figure 4.8E**). Together, these experiments support the conclusion that mutation at residues E126K and E157K specifically reduces neutralization by TS antibodies elicited by DENV1 vaccination.

Furthermore, the reduced sensitivity of variant E126K/E157K to immune sera from DENV1, but not DENV2, vaccinated individuals was not an artifact of changes in the structural dynamics of the virion, the efficiency of virion maturation, gross changes in the antigenic structure of the virion, or antigen excess in the neutralization assay.

4.4.4 Prevalence of an epitope recognized by TS antibodies present in the immune sera of DENV1-vaccinated subjects.

E126 and E157, we next performed longitudinal and cross-sectional studies of immune sera obtained from individual recipients of a DENV1 live-attenuated vaccine candidate (310). Participants in this vaccine study were administered vaccine on days 0 and 180, as described previously (310). Neutralization studies with sera from three subjects collected at days 28, 42, 180, 208, and 222 post-vaccination were performed using WT DENV1, WT DENV2, and DENV1 E126K/E157K RVPs. These three subjects were selected for study because they represented both high and low neutralizing antibody responses among participants in the phase I study. Dose response curves and summary NT50 values of sera from longitudinally tested subjects are shown in **Figure 4.9**. As anticipated, sera from post-vaccination samples neutralized DENV1 more efficiently than DENV2. In each instance, neutralization activity declined over time and was not boosted significantly by a second dose of vaccine, consistent with previous reports (**Figures 4.9 and 4.10**) (310).

Longitudinal analysis revealed that the sensitivity of the E126K/E157K variant to neutralization by DENV1-immune sera varied with time and among the three subjects tested. For Subject 43, the contribution of antibodies sensitive to mutations at positions

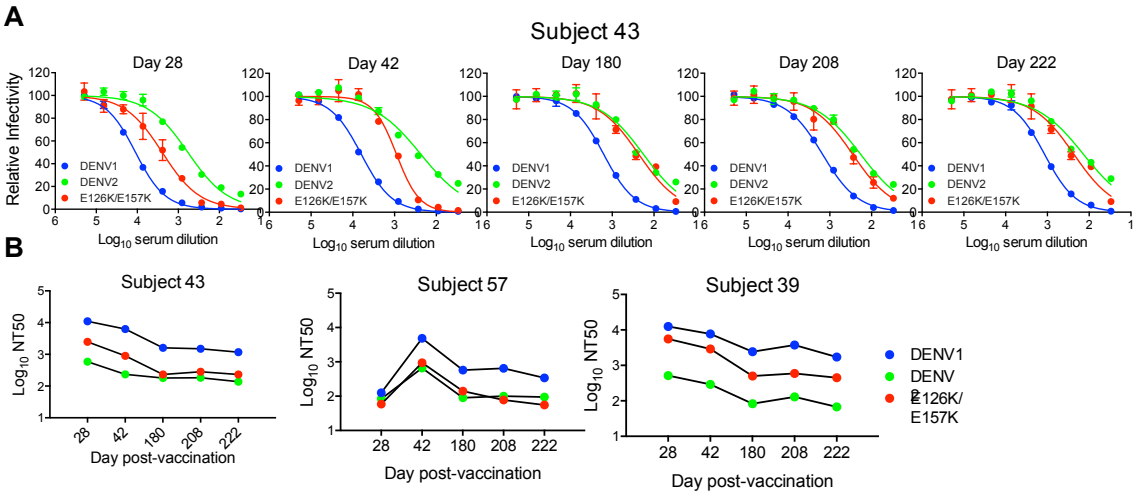
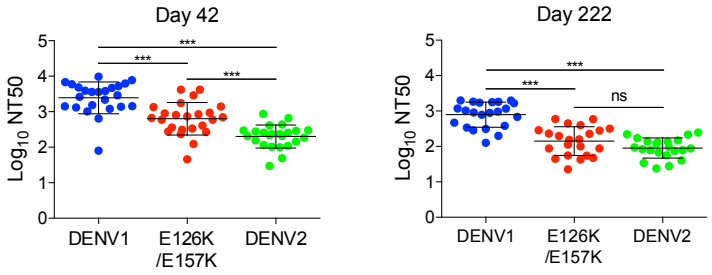


Figure 4.9 Longitudinal analysis of the effects of DENV1 E126K/E157K mutations on serum neutralizing activity. Sera collected from three DENV1 vaccine recipients at five times post-vaccination were tested for a capacity to neutralize WT DENV1, DENV2, and DENV1 E126K/E157K RVPs. (**A**) Dose-response curves for immune sera from one subject are shown. Error bars represent the standard error of duplicate infections. (**B**) NT50 values for each curve were determined by nonlinear regression analysis using Prism software (GraphPad), and are summarized for the three subjects.

A



B

	Day 42				Day 222			
	NT50			E126K+E157K /DENV2 fold difference	NT50			E126K+E157K /DENV2 fold difference
Subject	DENV1	E126+ E157K	DENV2		DENV1	E126+ E157K	DENV2	
32	1315	235	100	2.4	383	55	41	1.4
33	2913	1407	439	3.2	469	196	120	1.6
35	5974	1766	218	8.1	1024	225	78	2.9
36	3600	593	103	5.8	686	101	28	3.7
37	4675	1357	867	1.6	not available			
38	6793	4165	277	15.0	1664	591	130	4.5
39	7698	2893	291	10.0	1728	448	68	6.6
40	5255	812	156	5.2	1967	319	78	4.1
41	642	311	298	1.0	126	47	34	1.4
43	6246	898	233	3.8	1167	231	137	1.7
44	2628	695	256	2.7	1178	286	97	2.9
45	2178	593	116	5.1	966	398	75	5.3
46	1212	46	185	0.2	909	45	83	0.5
47	3812	736	145	5.1	779	159	88	1.8
49	1432	848	302	2.8	890	112	149	0.8
50	1384	124	30	4.2	284	23	24	0.9
51	80	278	49	5.6	199	87	56	1.6
52	3890	267	206	1.3	2003	87	248	0.4
53	1530	335	109	3.1	1813	282	172	1.6
55	3605	411	208	2.0	1834	154	217	0.7
56	9671	4203	414	10.2	1781	598	222	2.7
57	4831	941	661	1.4	341	55	94	0.6
58	1021	362	256	1.4	311	43	138	0.3
60	1415	664	234	2.8	not available			

Figure 4.10 Cross-sectional analysis of the contribution of the E126K/E157K epitopes on TS-neutralization. Sera from an additional 21 DENV1 vaccine recipients collected on days 42 and 222 (when available) were tested for their capacity to neutralize WT DENV1, DENV2 and DENV1 variant E126K/E157K RVPs. (**A**) The mean neutralization potency (Log NT50) of the sera against each virus is shown; error bars represent one standard deviation of the mean. ***p<0.0001; ns, p=0.15. (**B**) The neutralization titers (NT50) of the sera against WT DENV1, DENV2 and DENV1 E126K/E157K RVPs are presented. The fold-difference in sensitivity between DENV1 variant E126K/E157K RVPs and DENV2 RVPs was determined by the equation (NT50 DENV1 E126K/E157K)/(NT50 DENV2). Red shading indicates a <3 fold difference, yellow shading corresponds to a 3-6 fold difference, and green shading reflects a >6 fold difference.

E126 and E157 increased with time post-vaccination. By day 222, DENV2 and DENV1 E126K/E157K RVPs were neutralized to an equivalent degree (**Figure 4.9A and 4.9B**). The reduced sensitivity of DENV1 E126K/E157K RVPs to neutralization was observed at earlier time points in the sera of Subject 57, suggesting the TS-antibody response in this individual was focused early on epitopes impacted by mutating these two residues (**Figure 4.9B**). In contrast, while the sensitivity of DENV1 E126K/E157K RVPs to neutralization was reduced in comparison to WT at all time points sampled from Subject 39 (from 2-6 fold), mutation of these two residues alone did not ablate the entire TS response (**Figure 4.9B**).

To better understand the contribution of epitopes defined by the E126K and E157K mutations in the TS response to the DENV1Δ30 vaccine in a larger sample set, we assayed sera collected from an additional 21 DENV1 vaccine recipients at two time points post-vaccination (day 42 and 222; day 222 was collected 42 days after the second vaccine dose was administered). Antibody dose-response curves were generated for each sample using WT DENV1, WT DENV2, and DENV1 E126K/E157K RVPs as described above. The NT50 values calculated from this cross-sectional study are presented in **Figure 4.10A and B**. As expected, vaccine sera most efficiently neutralized DENV1; DENV1 E126K/E157K and DENV2 RVPs were significantly less sensitive to neutralization at both time points studied ($p<0.0001$ for both comparisons) (**Figure 4.10A**). While comparisons of the neutralization titer against DENV1 E126K/E157K and DENV2 revealed differences at day 42 post-vaccination ($p<0.0001$), by day 222 no significant difference was observed ($p=0.15$).

On average, we observed a 3.5-fold decrease in neutralization titers measured with DENV1 RVPs between day 42 and day 222 post-vaccination. This decay was more rapid than the change in DENV2-reactive titers measured at these two time points (mean 2.4-fold decrease) ($p<0.01$). Of interest, the ability of DENV1 sera to neutralize the E126K/E157K variant reactive antibody declined most dramatically between days 42 and 222 (a mean 4.8-fold decrease). This decline in sensitivity to neutralization was more rapid that that observed with either DENV1 ($p<0.05$) or DENV2 ($p<0.0001$) RVPs. This data is in agreement with our interpretation that over time, antibodies in DENV1-immune sera become more focused on epitopes containing residues E126 and E157. On average, RVPs containing mutations at these positions become more difficult to neutralize with time post-vaccination.

Analysis of individual neutralization titers revealed the same pattern (**Figure 4.10B**). On day 42, the serum of roughly half the recipients neutralized DENV1 E126K/E157K RVPs less efficiently than WT DENV1 RVPs. The contribution of antibodies binding epitopes impacted by mutation of E126 and E157 was monitored as the difference in neutralization sensitivity of the DENV1 E126K/E157K mutant and DENV2 RVPs (highlighted in red, yellow, and green to indicate 0-3, 3-6, and >6 fold differences in NT50). In 46% of the day 42 samples tested, the neutralization titer of the E126K/E157K variant was found to be within 3-fold of the cross-reactive DENV2 neutralization titer (**Figure 4.10B**). By day 222 post-vaccination, 77% of the NT50 values obtained with the E126K/E157K variant were within 3-fold of the DENV2 neutralization titer, confirming

the trend observed in the longitudinal analysis described above. Together, these data identify a significant functional contribution of epitopes containing residues E126 and E157 in the TS DENV1 response that increases with time.

4.5 Discussion

Interpreting the serology of flaviviruses is hindered by extensive cross-reactivity and significant differences in the functional properties of antibodies that bind different epitopes on the virion (47, 48, 50-54). Several approaches have been employed to deconstruct the complexity of the polyclonal antibody response to DENV. Biochemical studies using recombinant proteins or subviral particles have identified mutations in the E protein that reduce recognition by antibodies in DENV immune sera in an ELISA or Western blot assay format (54, 324, 325). A limitation of this type of approach is that it does not account for the marked difference in neutralization potential among antibodies of varying specificity. A neutralization response driven by the contribution of a low concentration of potent neutralizing antibodies may be difficult to detect using biochemical studies.

The antibody repertoire elicited by DENV infection or vaccination has also been investigated using approaches that allow study of the functional properties of antibodies in immune sera. Exciting advances have been made towards understanding the DENV antibody repertoire through the analysis of the specificity of human mAbs (50, 59, 60). A strength of this approach is that it enables functional analysis of antibody specificities that make up the polyclonal immune response. While this approach reveals the specificity

of DENV-reactive memory B-lymphocytes, it is unknown how faithfully these methods capture the breadth of the polyclonal humoral immune response *in vivo*, and how screening bias impacts the mAbs selected for study. A recent study of 26 DENV-reactive human mAbs obtained from subjects immunized with the rDEN1Δ30 vaccine candidate studied herein identified only three mAbs that neutralize DENV (301). The functional specificity of the polyclonal DENV immune response has also been investigated by depletion of immune sera using recombinant proteins. These studies suggested antibodies that bind E-DIII are not a significant component of the neutralizing antibody response and play only a modest role in protection in the AG129 mouse model of DENV infection/disease (197, 326). This finding was confirmed using infectious clones encoding mutations in E-DIII (327).

The number of epitopes that contribute to the neutralizing activity of the polyclonal antibody response to DENV remains unknown. To date, insight into where neutralizing antibodies present in human sera bind DENV has been limited principally to negative data. The goal of the present study was to identify the epitopes recognized by neutralizing antibodies of the type-specific DENV1 response following vaccination. We undertook a mutagenesis approach to identify amino acid substitutions that conferred a reduction in sensitivity to neutralization by DENV1 immune sera, but not cross-reactive antibodies present in DENV2 sera. We created a library of DENV1 RVPs encoding mutations at residues predicted to be surface-accessible on the mature virion that differ between DENV1 and DENV2. We identified single amino acids in E-DI (E126) and E-DII (E157)

that when mutated together abrogated a large portion of the TS-neutralizing antibody response in 77% of vaccinated subjects.

That two amino acid substitutions were sufficient to significantly reduce the TS-antibody response in the majority of vaccinees was a surprise. These data suggest the TS-antibody response is remarkably focused. Similar conclusions were reached in studies of the complexity of the polyclonal antibody response to HIV-1 and influenza (328-331). Residues E126 and E157 are very conserved among DENV1 viruses; the mutations identified in our study were found only once (E126K) or not at all (E157K) among 1,398 DENV1 sequences available for *in silico* analysis (332). Thus, the ability of DENV1 to escape from neutralization by mutation may be limited by the functional pressure of cross-reactive antibody and a substantial fitness cost. A more detailed analysis of the functional consequences of mutations at these residues is underway.

While the TS-immune response of a majority of volunteers in our study was focused significantly on epitopes affected by mutations at E126 and E157, these changes had a somewhat reduced impact on the potency of immune sera from five volunteers. This suggests that additional residues are involved in the fine specificity of the response and will require further study. Limited secondary screening with a subset of the panel of DENV1 variants identified residue 203 as a significant contributor to TS-neutralization patterns of Subject 38 (**Figure 4.11**), but not the others. As this residue is located within 13 Å from residue 126, it is possible that mutations at this position impact recognition of the same or overlapping epitopes in E-DII.

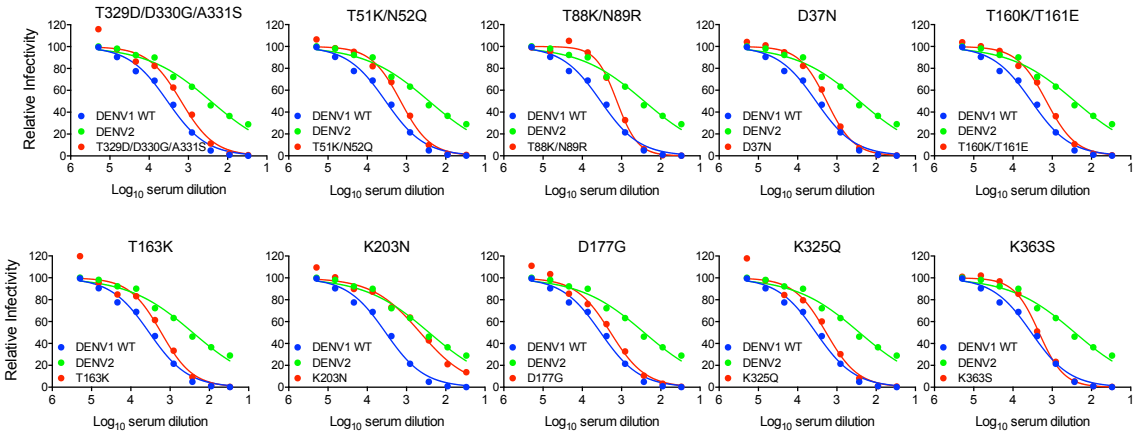


Figure 4.11 Neutralization of additional DENV1 variants by sera from DENV1 vaccine recipients. While the TS-immune response of a majority of volunteers in our study was focused significantly on epitopes affected by mutations at E126 and E157, these changes had a reduced impact on the potency of immune sera from five volunteers (Subjects 36, 38, 39, 40, and 45). Secondary screening of day 222 sera from these subjects was performed with a panel of ten of DENV1 variants shown to modestly decrease the potency of the DENV1 pooled serum (**Figure 4.4**). Only a role for mutant K203N in modulating the neutralization sensitivity of DENV1 immune sera of Subject 38 was identified as significant using our screening metric (<3-fold difference in NT50 between variant K203N and DENV2, n=2). Antibody-dose response curves from a representative screening study are displayed.

While, to our knowledge, residues 126 and 157 on DENV1 E protein have not yet been identified in neutralization escape studies with DENV mAbs, recent studies have identified a complex epitope in proximity to E157. Studies of the human anti-WNV mAb CR4354 identified an epitope at the junction of E-DI and E-DII that exists only on the intact virion, and not on soluble E protein (145). Four human mAbs have since been characterized that bind in the same region. The footprint of the TS DENV1 mAb DENV HM14c10 has been solved using cryo-electron microscopy reconstruction and has confirmed the discontinuous structure of the epitope and its similarity to CR4354 (58, 300). Neutralization escape mutants have been identified for HM14c10 as well as three additional human mAbs thought to recognize quaternary epitopes on DENV; each escape variant was mutated around the DI-II hinge region and within the CR4354 footprint (57, 300). The E157K mutation identified in our study is close to the hinge region. However, it remains unclear if antibodies that bind DENV1 at this location engage a quaternary epitope similar to CR4354, as this residue falls just outside the DI-DII hinge epitopes defined by the footprints of WNV CR4354 or DENV HM14c10. Furthermore, the 12 surface-exposed residues that differ between DENV1 and DENV2 present in the published DI-DII hinge epitope footprints did not themselves markedly reduce sensitivity to neutralization by DENV1 sera in this screen.

The dense arrangement of E proteins on the virion complicates our understanding of antibody recognition. Many well-characterized epitopes are not predicted to be accessible for antibody binding using existing static models of the mature DENV structure (54, 140, 171). For example, antibodies that bind the E-DII fusion loop are a significant component

of the CR repertoire produced by mouse and human following flavivirus infection, yet bind an epitope not predicted to be accessible for antibody recognition (54). The accessibility of cryptic epitopes like the DII-FL has been shown to be governed by several factors (32, 138). Viruses exist as an ensemble of structures at equilibrium (reviewed by (322, 333)). "Breathing" of envelope proteins incorporated into the flavivirus virion has been shown to alter epitope accessibility and sensitivity to neutralization by monoclonal and polyclonal antibody (138, 173, 334). In addition, many antibodies are sensitive to the maturation state of the virus particle (32). Engineered mutations or naturally occurring variation have the potential to alter the heterogeneity or dynamics of the virion, and therefore may impact epitope exposure. While we have identified amino acids that contribute to the type-specific recognition of the DENV1 Western Pacific strain, epitopes containing these amino acids may play a reduced or enhanced role in the context of other viral E protein sequences. Of interest, ~30% of the vaccine recipients studied within neutralize a related DENV1 strain 16007 more efficiently than the WP strain used in the DENV1 vaccine candidate (data not shown). This enhanced recognition may reflect differences in the "breathing" of these two viruses that alter the accessibility of epitopes on the virus particle, as suggested in a recent study (172). As our analysis was focused on surface accessible differences between the DENV1 and DENV2 components of the vaccine, it is possible that amino acid variation among DENV1 strains and differences in the extent of "viral breathing" (and therefore epitope exposure) will impact the pattern of type-specific recognition of different DENV1 strains. It remains to be determined whether the two resides characterized within will be useful as a generalizable signature of a type-specific humoral response for all DENV1 viruses.

Evaluating the contribution of this structural complexity towards neutralization sensitivity is a critical component of understanding the antigenic surface of flaviviruses and how this varies among strains within and between serotypes.

The identification of major targets of the TS-neutralizing antibody response to DENV1 vaccination represents an important step toward a more complete understanding of the humoral immune response to DENV. Whether epitopes including residues E126 and E157 contribute significantly to the neutralizing antibody response to natural infection or vaccination with all four DENV serotypes remains to be determined. The four serotypes of DENV share a 63% or greater amino acid identity and presumably the same overall virion structure. The epitopes engaged by TS-neutralizing antibodies elicited by infection of other DENV serotypes are unknown. Of note, the introduction of the reciprocal mutations at positions K126 and K157 of DENV2 NGC did not markedly shift the neutralization curve of DENV2 immune sera obtained from vaccine recipients (**Figure 4.12**). These data suggest that antibodies that contribute to the TS-neutralizing antibody response may bind distinct epitopes on all four DENV serotypes. Beyond the identification of significant epitopes in DENV, the data and experimental approaches described within provide insight into mutagenesis approaches for deconstructing the functional components of the polyclonal DENV antibody response and will be used to guide future studies on the functionally important epitopes involved in the neutralization of the other three DENV serotypes.

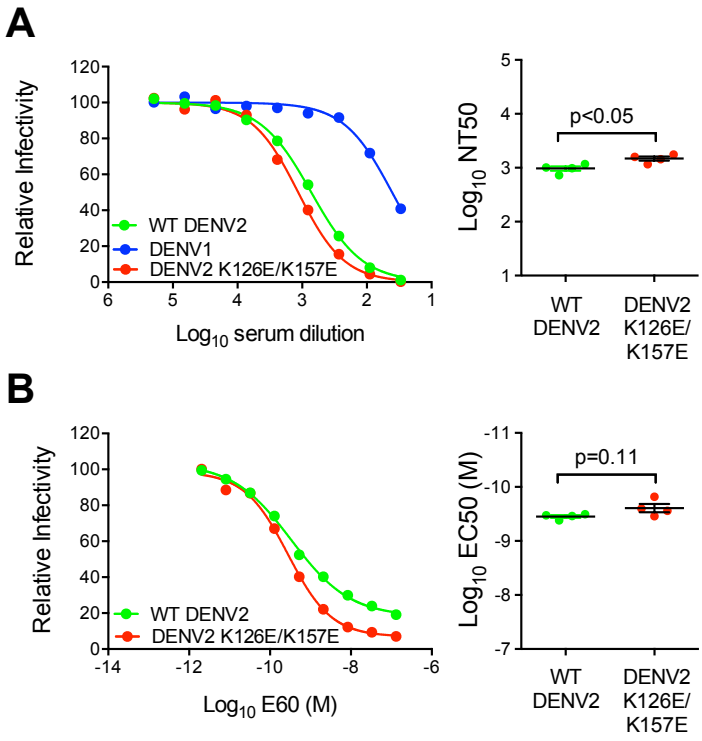


Figure 4.12 Effect of mutations at residues 126 and 157 on DENV2 RVPs. To test whether the residues 126 and 157 are targets of TS antibodies in DENV2 sera, a DENV2 NGC variant was constructed containing the reciprocal mutations, K126E and K157E. (**A**) DENV2 K126E/K157E RVPs were tested for sensitivity to neutralization by pooled DENV2 sera. Representative dose-response curves are shown on the left; error bars represent the standard error of duplicate infections. NT50 values from four independent experiments are shown on the right and reveal a modest 1.5-fold increase in neutralization sensitivity of the variant ($p<0.05$). (**B**) DENV2 K126E/K157E was tested for sensitivity to neutralization by CR mAb E60. Representative dose-response curves are shown on the left; error bars represent the standard error of duplicate infections. NT50 values from four independent experiments are shown on the right, and reveal a similar 1.4-fold increase in sensitivity to neutralization compared to WT DENV2, though this difference did not reach statistical significance ($p=0.11$).

Chapter 5: Investigating antigenic differences between DENV1 strains that mediate recognition by antibodies in polyclonal sera.

5.1 Introduction

Phylogenetic analyses reveal that within each DENV serotype, strains additionally cluster into three to five distinct groups, or genotypes (335). Strains within a genotype exhibit less than 6% nucleotide sequence or 3% amino acid sequence variation in the E gene (74). DENV serotype 1 is divided into five genotypes that differ by up to 6-10% in their E gene (nucleotide) sequences and up to 4-5% in their E protein (amino acid) sequences (335-337). Despite the sequence diversity between genotypes, it has generally been accepted that following primary infection, immunity develops against all of the strains of the infecting serotype. However, DENV genotype- and strain- dependent differences in neutralization sensitivity have recently become a focus of investigation, and both monoclonal antibodies (mAbs) and polyclonal sera have been characterized that have genotype-specific differences in neutralization potency (47, 48, 172, 178, 216, 307, 338).

Genotype- or strain-dependent differences in an antibody's neutralization potency may be due to sequence variation that occurs within the antibody's binding footprint and affects the binding energetics between the antibody and its epitope (47, 172, 178, 216, 307). Alternatively, differences in potency may be unrelated to a change in binding energetics (339, 340). Rather, amino acid variation that occurs outside of an epitope can indirectly affect neutralization potency through changes in epitope exposure (341). Two factors that

have an effect on flavivirus epitope exposure are virion maturation state and viral structural dynamics (32, 138). While virion maturation state has been shown to modulate the potency of many mAbs and cross-reactive polyclonal antibodies (pAbs) in DENV sera (32-34, 226), it's impact on the potency of the type-specific, neutralizing antibody response to DENV is minimal (33, 261).

Virion structural dynamics or viral "breathing" can affect flavivirus epitope exposure by altering the structural conformations sampled by dynamic E proteins (138). While the impact of virion structural dynamics on the potency of the human polyclonal antibody response to DENV is not known, viral breathing has been shown to impact the potency of some mAbs in a strain-dependent manner (47, 172, 178). For example, a study characterizing a panel of DENV1 mAbs found a marked reduction in neutralization potency against DENV1 strain WP compared to DENV1 strain 16007 for mAb E111 (47). Sequence variation within the E111 epitope on DIII did not fully explain the difference in neutralization sensitivity. Domain III of strains WP and 16007 differ by only two amino acids, at residues that did not match with the mAb epitope-mapping data. Notably, structural analysis of E111 revealed that its epitope was not predicted to be accessible to antibody binding on the cryo-EM reconstruction of DENV (172). Further experiments with E111 revealed that its neutralization potency increased with time and temperature of incubation of the immune complexes prior to addition to cells; this is consistent with previous studies demonstrating that cryptic epitopes may become available for antibody recognition in a time- and temperature-dependent fashion due to viral breathing (138, 173). However, the extent of the time- and temperature-dependent

increase in sensitivity to E111 neutralization was strikingly strain-dependent (172). Neutralization of WP was greatly enhanced by the increased incubation times and temperatures, while strain 16007 had more modest increases in sensitivity, presumably because the epitope was available for binding on 16007 at standard assay conditions or because 16007 virions more frequently sample conformations exposing 16007. The results from these studies suggest that DENV1 strain 16007 samples different or a broader range of conformations or samples conformations at a different frequency than DENV1 strain WP at steady-state, standard assay conditions; however, WP may sample alternate conformations that expose the E111 epitope following exposure to increased temperature and time.

The difference in the neutralization potency of E111 against 16007 and WP was mapped to a single amino acid difference between these strains (K. Dowd and T. Pierson, unpublished data). When E protein residue 204 was mutated in WP from a K to an R, WP variant K204R demonstrated greatly enhanced sensitivity to neutralization by E111, similar to the level of sensitivity displayed by 16007. When the reciprocal mutation was introduced into 16007, 16007 variant R204K was significantly more resistant to E111, similar to levels observed for WT WP. Notably, residue 204 is located in E-DII outside of the E111 epitope in DIII. These data suggest that residue 204 may modulate the structural conformations sampled by WP and 16007. Notably, with the exception of strain 16007, residue K204 is highly conserved among DENV1 strains.

The residues identified in Chapter 4 as a focus of the type-specific neutralizing antibody response to DENV1 vaccination based on the WP strain (residues E126 and E157) are highly conserved among DENV1 viruses (as assessed from over 1300 sequences available for analysis) (332). Despite the conservation of E126 and E157, strain-dependent differences in structural dynamics could impact the exposure of epitopes that include these residues. Additionally, it is unknown what additional residues make up the epitope(s) containing E126 and E157, and whether they are conserved between DENV1 strains. In this study, we investigated neutralization of heterologous DENV1 strains by sera from recipients of the NIH, WP-derived, DENV1 monovalent vaccine (14). We sought to determine whether the type-specific antibody specificities in polyclonal sera that mediate neutralization of WP are important in neutralization of heterologous DENV1 strains. We characterized the effect of mutations E126K/E157K on heterologous DENV1 strains, and observed strain-dependent differences in their impact on sensitivity to DENV1 sera that are mediated by differences in virion structural dynamics. This study provides insight into strain-dependent differences in recognition by polyclonal antibodies and highlights the complexities involved in understanding the contribution of individual residues to antibody recognition.

5.2 Materials and Methods

5.2.1 Cell lines

HEK-293T cells and Raji-DCSIGNR cells were maintained at 37°C and 7% CO_2. HEK-293T cells were passaged in complete Dulbecco's modified Eagle medium (DMEM)

containing Glutamax (Invitrogen, Carlsbad, CA), supplemented with 7.5% fetal bovine serum (FBS) (HyClone, Logan, UT) and 100 U/ml penicillin-streptomycin (PS) (Invitrogen, Carlsbad, CA). Raji-DCSIGNR cells were passaged in RPMI-1640 medium containing Glutamax (Invitrogen, Carlsbad, CA), supplemented with 7.5% FBS and 100 U/ml PS.

5.2.2 DENV immune sera

Sera from recipients of phase I studies of candidate DENV1 or DENV2 monovalent vaccines were obtained for study. Pooled serum samples were from two (DENV1) or three (DENV2) vaccine recipients collected 2-3 years post-vaccination. The individual serum samples from 22 recipients of a DENV1 vaccine were collected on day 222 post-vaccination (310).

5.2.3 Ethics Statement

Clinical studies were conducted at the Center for Immunization at the Johns Hopkins Bloomberg School of Public Health under an investigational new drug application reviewed by the United States Food and Drug Administration. The clinical protocol and consent form were reviewed and approved by the NIAID Regulatory Compliance and Human Subjects Protection Branch, the NIAID Data Safety Monitoring Board, the Western Institutional Review Board, and the Johns Hopkins University Institutional Biosafety Committee (ClinicalTrials.gov identifiers; NCT00473135, NCT00920517). Written informed consent was obtained from each participant in accordance with the

Code of Federal Regulations (21 CFR 50) and International Conference on Harmonisation guidelines for Good Clinical Practice (ICH E6).

5.2.4 Plasmids

Plasmids encoding a WNV sub-genomic replicon and the structural genes of DENV1 genotype 4 strain Western Pacific-74 (WP), DENV1 genotype 2 strain 16007, and DENV2 strain New Guinea C (NGC) have been described previously (34, 138, 226, 259-261). For the construction of a plasmid encoding the structural genes of DENV1 genotype 1 strain TVP2130, the structural genes were isolated from virus received from Dr. Michael Diamond (Washington University School of Medicine, St. Louis, Missouri) (47) through reverse transcription polymerase chain reaction (RT-PCR). The structural genes were cloned into the expression vector pcDNA6.2 (Invitrogen, Carlsbad, CA) and its sequence matched the CprME protein sequence of GenBank entry BAN63086.1. CprME variants were produced by site-directed mutagenesis using the Quikchange Mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions. All plasmids used in this study were propagated in Stbl2 bacteria grown at 30°C (Invitrogen, Carlsbad, CA).

5.2.5 RVP production

DENV RVPs were produced by complementation of a WNV replicon with plasmids encoding the structural genes of DENV as described previously (138, 259, 261). Briefly, pre-plated HEK-293T cells were transfected with plasmids encoding the WNIIrepG/Z

replicon and DENV CprME in a 1:3 ratio by mass, using Lipofectamine LTX or Lipofectamine 3000 (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's instructions. RVPs were produced at 30°C in the presence of a low-glucose formulation of DMEM containing 25 mM HEPES (Invitrogen, Carlsbad, CA), 7% FBS, and 100 U/ml PS. RVP samples were collected at 72, 96, 120, or 144 hours post-transfection, filtered using a 0.22μm syringe filter, and stored at -80°C. The infectious titer of RVPs was determined using Raji cells that express the flavivirus attachment factor DCSIGNR as described previously (130, 259). Following infection of Raji-DCISIGNR cells with serial dilutions of RVP samples, cells were incubated at 37C°C for two days and scored for infection as a function of GFP expression by flow cytometry.

5.2.6 Neutralization assays

DENV RVP stocks were diluted and incubated with serial dilutions of mAb or serum for one hour at room temperature prior to the addition of Raji-DCSIGNR cells, unless specified otherwise. Infections were carried out at 37°C for 48 hours and infectivity was scored as the fraction of GFP-expressing cells determined using flow cytometry. Antibody-dose response curves were analyzed using non-linear regression analysis (with a variable slope) (GraphPad Software, San Diego, CA). Data are expressed as the concentration of antibody (EC50) or serum dilution (NT50) required to reduce infection by half.

5.2.7 Statistical analysis

Statistical analyses were performed using Prism software version 6.0f for Mac OS X (GraphPad). Log EC50 or Log NT50 values were compared using Student's *t*-test when comparing two samples. For comparisons of more than two samples, Log NT50 values were compared by one-way ANOVA followed by Tukey's multiple comparisons test.

5.3 Results

5.3.1 Characterization of RVPs encoding the structural genes of additional DENV1 strains.

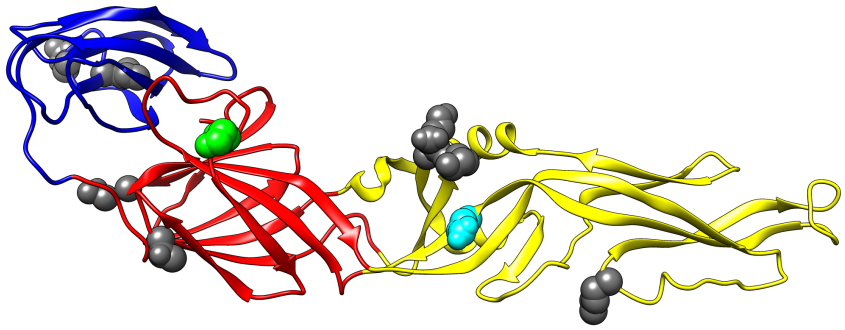
The DENV1 component of the NIH DENV vaccine candidate is derived from DENV1 strain WP (genotype 4) (14). We previously used RVPs of DENV1 strain WP to investigate the specificities of antibodies elicited by this vaccine (261). To investigate the specificities involved in polyclonal antibody recognition of other DENV1 strains, we selected two additional strains from heterologous genotypes for study: strain 16007 (genotype 2) and strain TVP2130 (genotype 1). The E protein of strain 16007 differs from that of WP by 13 amino acids (2.6% variation), while the E protein of strain TVP2130 differs from that of WP by 11 amino acids (2.2% variation) (**Figure 5.1**). Of note, residue 204, which was shown to mediate the differences in structural dynamics between strains WP and 16007 (K. Dowd and T. Pierson, unpublished data), is conserved between strains WP and TVP2130.

Following production of RVPs encoding the structural genes of DENV1 strains 16007 and TVP2130, we measured the sensitivity of these strains to neutralization by mAb

A

Region		DI	DII	DI		DII				DIII			stem				TM			
Residue #		37	88	155	180	202	203	204	225	339	345	351	397	432	436	439	461	472	478	484
Amino acid	16007	N	A	T	T	K	E	R	S	T	A	L	S	M	V	V	I	N	M	M
	WP	D	T	T	A	E	K	K	S	S	V	L	S	V	I	I	I	S	T	M
	TVP2130	D	A	S	A	K	E	K	T	T	V	V	T	V	V	I	V	S	T	L

B



C

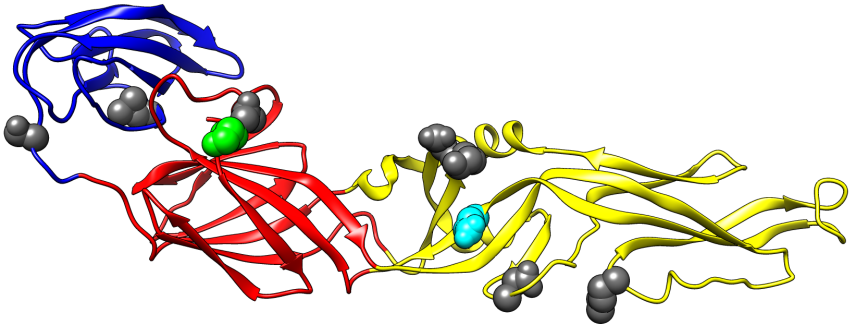


Figure 5.1 Differences in E protein sequence between DENV1 strains WP, 16007, and TVP2130. (A) Amino acids in the E protein of 16007 and TVP2130 that are different from the WP residue are highlighted in grey. Residue location within the E protein ectodomain (DI, DII, and DIII) or within the stem or transmembrane (TM) is indicated on the top line. **(B** and **C)** Amino acid differences between the E proteins of (**B**) strains WP and 16007 or **(C**) strains WP and TVP2130 are highlighted in dark grey on the crystal structure of the ectodomain of the E protein. Additionally, residue E126 is highlighted in cyan and residue E157 is highlighted in green. The E protein domains are colored in red (DI), yellow (DII), and blue (DIII).

E103. mAb E103 is a DENV1-type-specific antibody that binds a DIII epitope and has previously been shown, like E111, to neutralize strain 16007 more potently than strain WP (47). Likewise, we observed enhanced neutralization of strain 16007 (Log EC50 -11.45 M) compared to strain WP (Log EC50 -10.19 M; $p<0.0001$) (**Figure 5.2A**). Strain TVP2130 was also neutralized more potently than strain WP (Log EC50 -10.95, $p=0.0002$), though not as potently as 16007 ($p<0.0001$). This is in contrast to mAb E111, which had decreased inhibitory activity against TVP2130 relative to WP (172).

We additionally tested the DENV1 strains for their sensitivity to neutralization by serum samples pooled from recipients of NIH DENV1 or DENV2 monovalent vaccine candidates (317, 318). For these experiments, we employed the pooled DENV serum samples previously used in Chapter 4 (**Figures 4.2 and 4.4**). Notably, though antibodies in these sera were raised against strain WP, strain 16007 was significantly more sensitive to neutralization by the pooled DENV1 sera compared to strain WP (3.1-fold difference, $p=0.0001$) (**Figure 5.2B**). In contrast, there was no significant difference in neutralization sensitivity between strains WP and TVP2130 ($p=0.79$). When the cross-reactive antibodies in DENV2 pooled sera were tested for an ability to neutralize the three DENV1 strains, no significant difference in the mean NT50 values was observed ($p>0.75$ for WP vs. 16007 and for WP vs. TVP2130 comparisons) (**Figure 5.2C**). These data suggest that strain-dependent differences in structural dynamics may enhance the sensitivity of strain 16007 to neutralization by mAb E103 and DENV1 polyclonal sera in addition to mAb E111, but not to cross-reactive DENV2 sera. In contrast, while strain TVP2130 had enhanced sensitivity to E103 relative to strain WP, it did not reach the

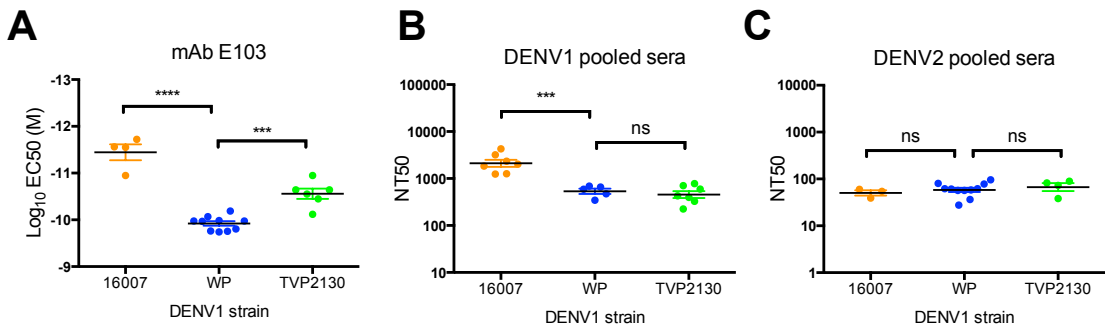


Figure 5.2 Neutralization sensitivity of DENV1 strains to polyclonal sera and monoclonal antibodies. (**A**) DENV1 strains were tested for their neutralization sensitivity to mAb E103. Mean EC50 values are shown for independent experiments for 16007 (n=4), WP (n=10) and TVP2130 (n=6). **(B and C)** DENV1 strains 16007, WP, and TVP2130 were tested for neutralization sensitivity to pooled serum samples collected from two and three recipients of the DENV1 and DENV2 monovalent vaccines, respectively. (**B**) The mean neutralization titers of the DENV1 sera are shown from independent experiments for strains 16007 (n=7), WP (n=5), and TVP2130 (n=7). (**C**) The mean neutralization titers of the DENV2 sera shown from independent experiments for 16007 (n=3), WP (n=11) and TVP2130 (n=4). For all panels, error bars represent standard error of the mean. ns, $p>0.05$;***$p<0.001$; ****$p<0.0001$.

level of sensitivity observed for strain 16007. Furthermore, strain TVP2130 did not display enhanced sensitivity to the DENV1 pooled sera.

5.3.2 Differential neutralization of DENV1 strains by sera from DENV1 vaccine recipients.

In order to further assess whether DENV1 sera raised against strain WP differentially neutralize heterologous DENV1 strains, we performed a screen using individual serum samples from recipients of the NIH DENV1 monovalent vaccine candidate. These serum samples were used previously in experiments described in Chapter 4 (**Figure 4.10**). For this screen, we employed sera collected on day 222 post-vaccination; 22 serum samples at this time point were available. DENV1 strains WP, 16007, and TVP2130 were tested for their sensitivity to neutralization by each of the 22 DENV1 serum samples. While the overall mean neutralization titers of the panel of sera were not significantly different against strain 16007 compared to strain WP ($p=0.22$) (**Figure 5.3A**), comparisons of individual neutralization titers revealed distinct patterns of neutralization. Serum samples from subjects 35 and 38 had decreased potency against strain 16007 (2.1-fold and 9-fold, respectively), whereas seven serum samples (32% of samples) had >2-fold increased potency against strain 16007 (**Figure 5.3B**). The remaining 13 serum samples (59%) neutralized strains 16007 and WP with NT50 values that differed by less than two-fold. The enhanced neutralization potency of almost one third of the sera against strain 16007 relative to the strain that elicited the response may reflect antigenic differences arising from structural dynamics.

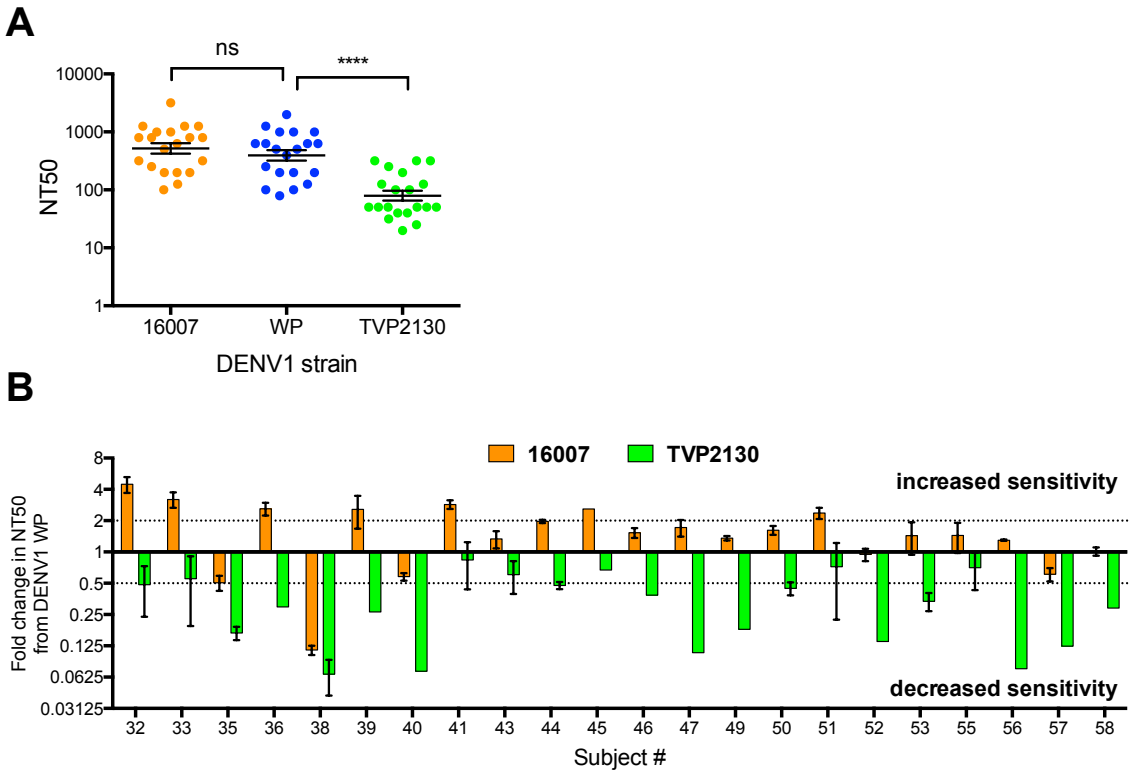


Figure 5.3 Differences in neutralization potency of DENV1 sera against strains 16007, TVP2130, and WP. (**A**) Mean neutralization titers for 22 serum samples obtained 222 days following monovalent DENV1 WP vaccination against DENV1 strains WP, 16007, and TVP2130. Error bars represent one standard deviation of the mean. ****p<0.0001; ns, p=0.22. (**B**) Fold difference in neutralization titer between strain WP and either strain 16007 (orange) or strain TVP2130 (green) obtained from pairwise experiments for each of the 22 serum samples. Values greater than 1 indicate increased neutralization sensitivity compared to strain WP while those less than 1 indicate decreased neutralization sensitivity. Mean NT50 values from 1-3 experiments for each serum sample are shown; error bars represent standard error of the mean.

In contrast to the results obtained for strain 16007, the mean neutralization titers against strain TVP2130 were 5.3-fold lower than those against strain WP ($p<0.0001$) **(Figure 5.3A**). Furthermore, comparisons of individual neutralization titers reveal that 18 serum samples (82%) had >2-fold decrease in potency against strain TVP2130 compared to strain WP, while the remaining 4 serum samples (from subjects 41, 43, 45, and 55) neutralized strains WP and TVP2130 equivalently. These data indicate that DENV1 sera obtained following monovalent vaccination with strain WP display strain-dependent differences in neutralization potency.

5.3.3 Characterization of E126K/E157K variants of DENV1 strains 16007 and TVP2130.

As mentioned, residues E126 and E157, which were determined to be the focus of neutralizing antibodies against strain WP following monovalent DENV1 vaccination, are conserved among DENV1 strains. To test whether these residues are also important for the neutralization sensitivity of heterologous DENV1 strains to DENV1 WP vaccine sera, we produced E126K/E157K variants for DENV1 strains 16007 and TVP2130. To ensure that the E126K/E157K mutations introduced into strains 16007 and TVP2130 did not result in changes in overall antigenicity of the E protein, variants were first characterized for sensitivity to neutralization by control antibodies that target epitopes outside those defined by E126/E157, and that were previously used to characterize the WP variant (**Figure 4.7 and 4.8**). First, the variants were tested for sensitivity to neutralization by pooled DENV2 sera. Mean NT50 values against 16007 variant E126K/E157K were not significantly different from those against WT 16007 (<2-fold different; $p=0.07$; $n=3$)

(**Figure 5.4A**). Likewise, we did not observe a large difference in NT50 values against TVP2130 variant E126K/E157K compared to WT TVP2130 (2-fold; p=0.053; n=3) (**Figure 5.4B**). Second, the variants were tested for sensitivity to neutralization by mAb E60, a cross-reactive, maturation state-sensitive antibody that binds the DII fusion loop. The EC50 values of E60 against 16007 variant E126K/E157K were not significantly different from those against WT 16007 (<2-fold different; p=0.61; n=3) (**Figure 5.4C**). Likewise, EC50 values of E60 against TVP2130 variant E126K/E157K and WT TVP230 were not significantly different (<2-fold different; p=0.38; n=4) (**Figure 5.4D**). Third, the variants were tested for their sensitivity to neutralization by DIII-binding mAb E103. EC50 values of E103 against 16007 variant E126K/E157K and WT 16007 were not significantly different (<2-fold different; p=0.07; n=3) (**Figure 5.4E**). Likewise, EC50 values of E103 against TVP2130 variant E126K/E157K and WT TVP2130 were not significantly different (<2-fold different; p=0.76; n=4) (**Figure 5.4F**).

Altogether, these results indicate that mutations E126K/E157K, when introduced into DENV1 strains 16007 or TVP2130, did not alter overall antigenicity of the E protein.

5.3.4 Effect of mutations E126K/E157K on neutralization of DENV1 strains 16007 and TVP2130 by DENV1 sera.

We next assessed the 16007 and TVP2130 E126K/E157K variants for sensitivity to neutralization by DENV1 sera. Although the WP E126K/E157K variant was previously shown to have significantly reduced sensitivity to the pooled DENV1 sera relative to WT WP (9.8-fold change, **Figure 4.6C and D**), the 16007 E126K/E157K variant displayed similar neutralization sensitivity against this pooled sera compared to WT 16007 (1.4-

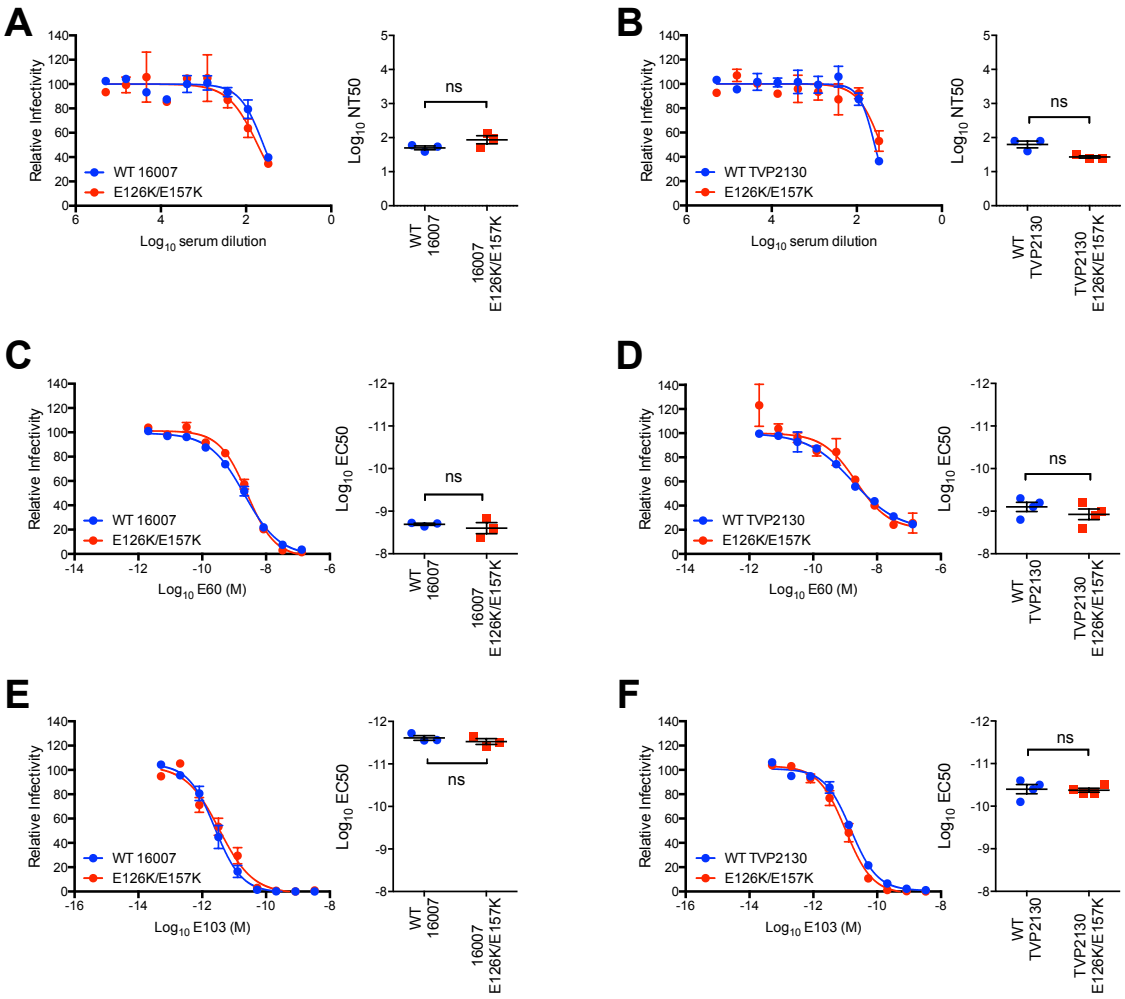


Figure 5.4 Characterization of E126K/E157K variants of DENV1 strains 16007 and TVP2130. E126K/E157K variants of DENV1 strains 16007 and TP2130 were compared to the WT virus for sensitivity to neutralization by DENV2 pooled sera, mAb E60, and mAb E103. Within each panel, representative dose response curves are shown of the left; error bars represent the range of duplicate infections. Mean NT50 or EC50 values from independent experiments are shown on the right; error bars represent the standard error of the mean; ns, p>0.05. (**A** and **B**) Neutralization potency of DENV2 pooled sera against (**A**) 16007 E126K/E57K and (**B**) TVP2130 E126K/E157K. (**C** and **D**) Neutralization potency of mAb E60 against (**C**) 16007 E126K/E57K and (**D**) TVP2130 E126K/E157K. (**E** and **F**) Neutralization potency of mAb E103 against (**E**) 16007 E126K/E57K and (**F**) TVP2130 E126K/E157K.

fold change, p=0.10, n=4) (**Figure 5.5A and B**). In contrast, the TVP2130 E126K/E157K variant displayed significantly reduced neutralization sensitivity compared to WT TVP2130 (5.6-fold change, p=0.014, n=4) (**Figure 5.5C and D**).

We next assessed neutralization of 16007 and TVP2130 E126K/E157K variants by individual DENV1 serum samples. For this screen, we chose a random subset (13 samples) of the 22 individual serum samples studied in **Figure 5.3**. Each serum sample was measured for neutralization activity against the E126K/E157K variant of 16007 or TVP2130 in parallel with WT DENV1 strains WP, 16007, and TVP2130, and DENV2 strain NGC. DENV2 neutralization titers were used as a reference to quantify the type-specific component of the serum neutralizing activity. In **Figure 5.6A**, the fold differences in neutralization titer between DENV2 and each WT DENV1 strain are shown, indicating the extent of the type-specific neutralization of each serum sample against each DENV1 strain. Analysis of the majority of the DENV1 sera revealed significant type-specific patterns of neutralization against DENV1 strain WP, with an average 9.4-fold increase in titer against WP compared to DENV2 (**Figure 5.6A**). Of the 13 samples tested, 10 had >6-fold increase in titer between WP and DENV2. The type-specific titers against DENV1 strain 16007 were even more pronounced than those against WP, with an average 18.4-fold increase in titer relative to DENV2 (**Figure 5.6A**). As with WP, 10 of the samples neutralized 16007 with >6-fold greater potency compared to DENV2, with the remaining three samples displaying a 3- to 5-fold increase in potency. In contrast, the type-specific titers against DENV1 strain TVP2130 were much lower, with an average 4.6-fold increase in titer relative to DENV2 (**Figure 5.6A**). Of the

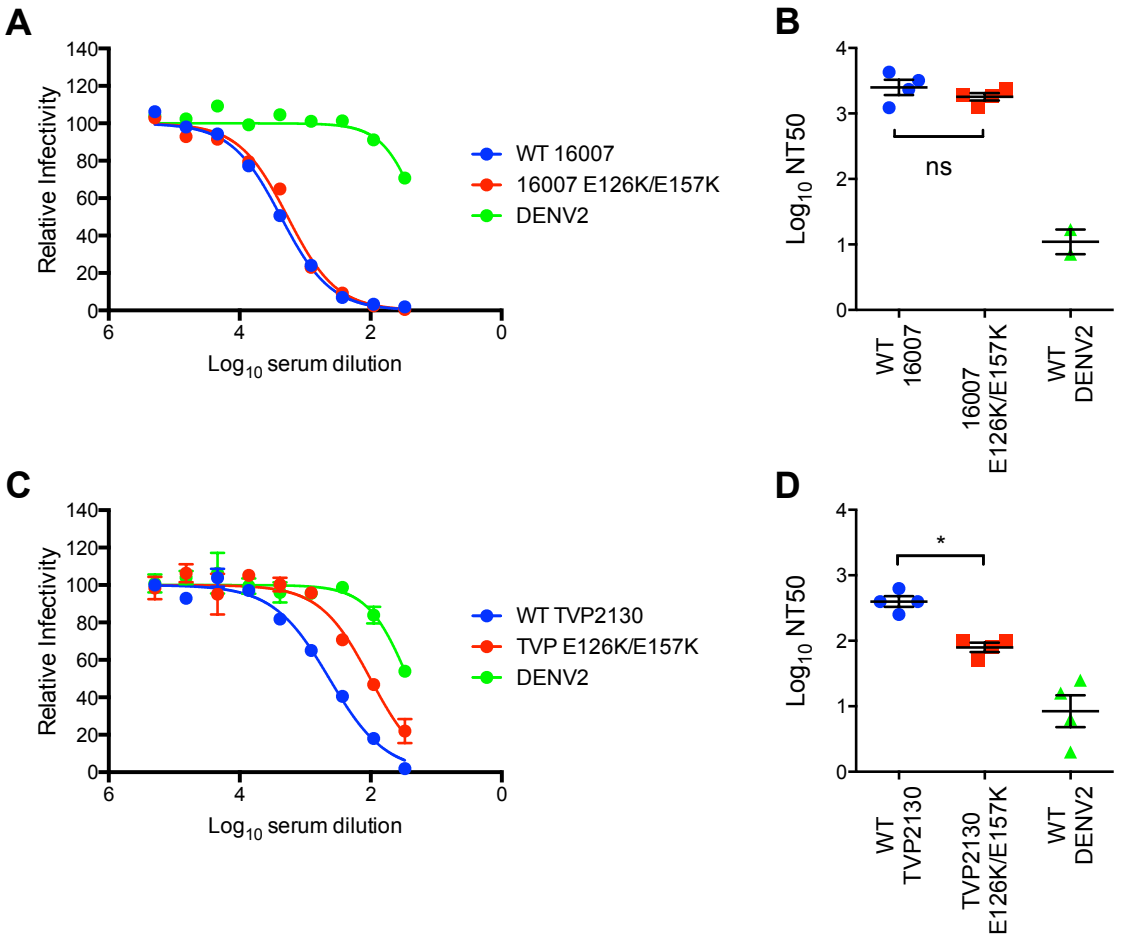


Figure 5.5 Effect of mutations E126K/E157K on the sensitivity of strains 16007 and TVP2130 to neutralization by DENV1 pooled sera. (A and **B)** WT 16007 and 16007 E126K/E157K were tested for sensitivity to neutralization by DENV1 pooled sera (from **Figure 5.2B**). (**A**) A representative dose-response curve is shown. Error bars represent the range of duplicate infections. (**B**) Mean NT50 values are shown from 4 (WT 16007 or 16007 E126K/E157K) or 2 (DENV2) independent experiments; error bars represent the standard error of the mean; ns, p=0.10. **(C and D)** WT TVP2130 and TVP2130 E126K/E157K were tested for neutralization sensitivity to DENV1 pooled sera. (**A**) A representative dose-response curve is shown. Error bars represent the range of duplicate infections. (**B**) Mean NT50 values are shown from 4 independent experiments; error bars represent the standard error of the mean, *p=0.014.

A

Subject	Fold change in NT50: WT DENV1 strain /DENV2		
	WT WP /DENV2	WT 16007 /DENV2	WT TVP2130 /DENV2
32	9.4	22.1	2.7
33	3.9	3.8	1.6
35	13.1	15.8	3.8
38	12.8	5.2	3.5
41	3.7	3.1	2.9
43	8.5	11.8	4.2
44	12.1	13.5	5.5
45	13.0	96.6	16.3
46	10.9	10.6	1.4
50	11.9	16.3	4.6
51	3.5	5.0	2.9
53	10.5	18.5	3.1
55	8.5	16.4	7.0
mean	9.4	18.4	4.6

B

Subject	Fold change in NT50: DENV1 E126K+E157 variant /DENV2		
	WP variant /DENV2	16007 variant /DENV2	TVP2130 variant /DENV2
32	1.4	15.3	1.0
33	1.6	3.3	0.9
35	2.9	6.9	2.0
38	4.5	6.0	3.1
41	1.4	2.3	0.7
43	1.7	10.9	1.4
44	2.9	8.8	2.0
45	5.3	80.2	9.3
46	0.5	2.0	0.5
50	0.9	7.5	1.5
51	1.6	4.1	1.7
53	1.6	11.8	1.7
55	0.7	5.3	1.6
mean	2.1	12.6	2.1

Figure 5.6 Sensitivity of DENV1 E126K/E157K variants to neutralization by DENV1-immune serum samples. DENV1 16007 and TVP2130 E126K/E157K variants were tested in parallel with WT 16007, WT TVP2130, WT WP, and WT DENV2 for sensitivity to neutralization by 13 DENV1-immune serum samples. Data from **Figure 4.10** for the WP E126K/E157K variant are included. (**A**) The fold difference in neutralization titer between each WT DENV1 strain and WT DENV2 is shown. Higher numbers indicate greater type-specific neutralization potency. (**B**) The fold difference in neutralization titer between each DENV1 E126K/E157K variant and WT DENV2 is shown. For both panels, red shading indicates a <3-fold difference, yellow shading a 3- to 6-fold difference, and green shading a >6-fold difference in titer.

13 serum samples, only two had a >6-fold increase in titer against TVP2130 compared to DENV2, while six had between a 3- to 6-fold increase in titer, and five had <3-fold increase in titer.

Figure 5.6B shows the extent of type-specific neutralization of each serum sample that was remained after introduction of the E126K/E157K mutations. In addition to the data obtained for E126K/E157K variants for strains 16007 and TVP2130, previously reported data for WP variant E126K/E157K (from **Figure 4.10)** are included for reference. As previously discussed, mutations E126K/E157K ablated the majority of the type-specific neutralization titers against DENV1 WP, such that the mean neutralization titers against this variant and DENV2 were within 2.1-fold. Of the 13 samples used in this study, 11 neutralized the WP E126K/E157K variant and DENV2 with similar potency (<3-fold difference in titer), and the remaining two samples displayed <6-fold difference. For TVP2130, as for WP, the E126K/E157K mutations ablated the majority of the type-specific neutralization titers, with an average 2.1-fold difference in neutralization titer between the TVP2130 variant and DENV2 (**Figure 5.6B)**. Twelve of the 13 samples had <3-fold difference in titer between the variant and DENV2, while the remaining sample retained a 9-fold difference. In contrast, the 16007 E126K/E157K variant was still neutralized in a type-specific manner by the majority of sera: seven samples had >6-fold difference in titer against the 16007 variant compared to DENV2, while four had a 3- to 6-fold difference, and only one sample had <3-fold difference (**Figure 5.6B)**. These results indicate that, in the context of DENV1 strains WP and TVP2130, mutations E126K/E157K ablate the majority of the type-specific neutralizing antibody response

elicited by a DENV1 vaccine. However, in the context of strain 16007, residues E126 and E157 do not play as large a role in polyclonal antibody recognition.

5.3.5 Impact of E protein residue 204 on neutralization of 16007 by DENV1 sera.

Although the antibodies in the DENV1 sera were raised against the WP strain, many of the serum samples more strongly neutralized DENV1 strain 16007. Our hypothesis is that differences in structural dynamics increase the exposure of epitopes on 16007 that are cryptic on WP. Because the amino acid difference at residue 204 has previously been shown to mediate differences in structural dynamics between the two strains, we investigated the ability of DENV1 sera to neutralize 16007 variant R204K. Sera from subjects 32, 33, and 51, which were each more potent against 16007 compared to WP (**Figure 5.3**), were tested for neutralization potency against 16007 R204K. All three sera displayed reduced activity against 16007 R204K relative to WT 16007, similar to levels observed for WP (**Figure 5.7A-C**).

These results indicate that differences in structural dynamics mediated by residue 204 may contribute to the enhanced neutralization of 16007 by DENV1 sera. Alternatively, if residue 204 is part of epitopes recognized by antibodies in sera, the R204K mutation may be directly impacting antibody binding (antibody affinity). To test this hypothesis, we introduced a non-chemically conservative substitution at residue 204 in 16007: a change in charge from a basic R to an acidic D residue. Unlike R204K, the R204D substitution did not alter the sensitivity of 16007 to neutralization by mAb E111 (**Figure 5.7D and E**). We then tested the R204D variant for sensitivity to neutralization by DENV1 sera

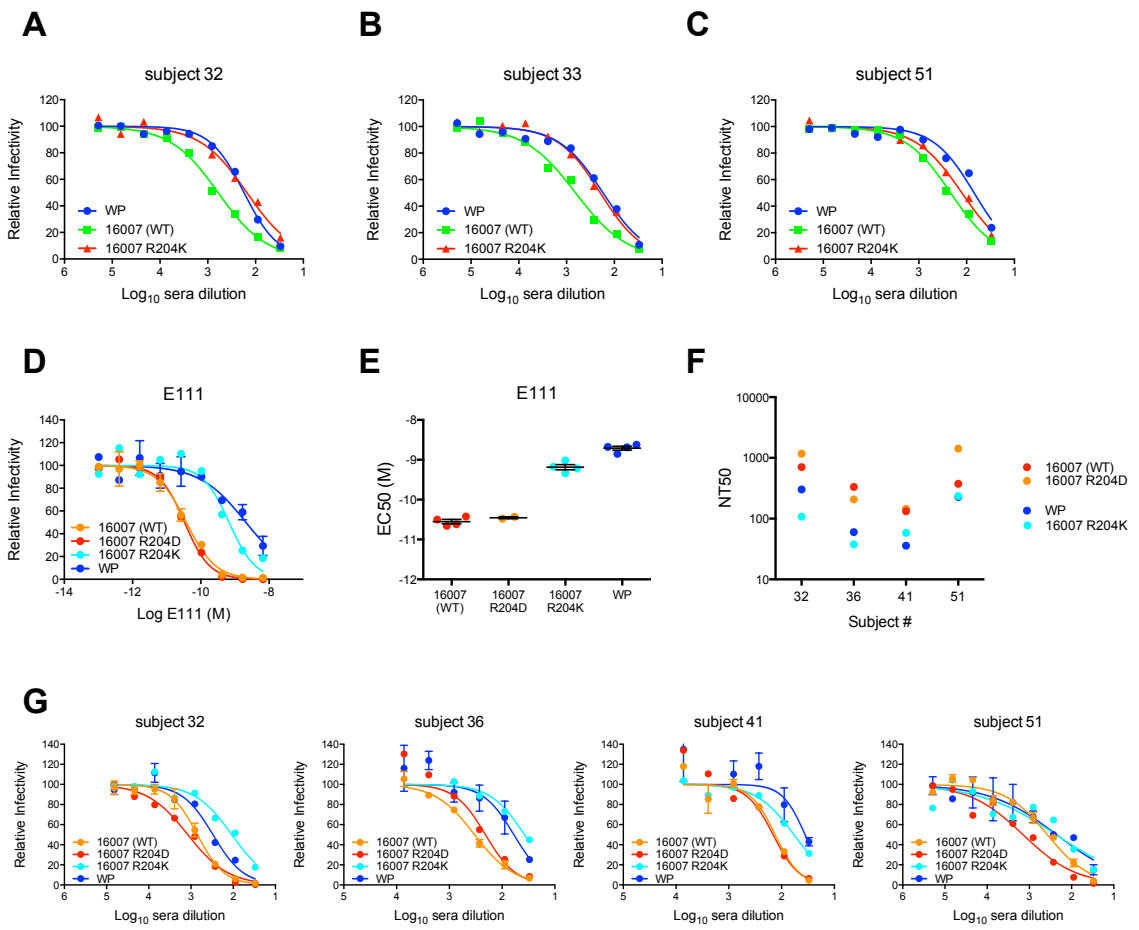


Figure 5.7 Impact of E protein residue 204 on neutralization of strain 16007 by DENV1 sera. (**A-C**) Sera from three DENV1 vaccine recipients were analyzed for their neutralization potencies against DENV1 strains WP, 16007, and 16007 variant R204K. Shown are the dose response curves for sera from (**A**) subject 32, (**B**) subject 33, and (**C**) subject 51. (**D** and **E**) mAb E111 was assessed for its neutralization potency against 16007 variants R204D and R204K, in addition to WT DENV1 strains 16007 and WP. (**D**) Shown is a representative dose response curve. Error bars represent the range of duplicate infections. (**E**) Shown are the mean E111 EC50 values from four (16007 WT, 16007 R204K, and WP) or two (16007 R204D) independent experiments. Error bars represent the standard error of the mean. (**F** and **G**) 16007 variants R204D and R204K and WT DENV1 strains 16007 and WP were assessed for their sensitivity to neutralization by four serum samples from DENV1 vaccine recipients. (**F**) Summary of the NT50 values for sera from all four subjects. (**G**) Dose response curves for all four serum samples. Error bars represent the range of duplicate infections.

previously shown to neutralize 16007 more potently than WP. If the chemically conservative R204K mutation affects the potency of DENV1 sera by directly disrupting antibody interactions, then the R204D mutation would likely also impact antibody recognition by the DENV1 sera. However, 16007 variant R204D and WT 16007 displayed similar sensitivities to DENV1 sera (**Figure 5.7F and G**), in contrast to the reduced sensitivity of 16007 R204K relative to WT 16007. These results suggest that the amino acid difference at residue 204 between strains WP and 16007 may modulate virion structural dynamics and alter the exposure of epitopes targeted by neutralizing antibodies in polyclonal sera.

5.4 Discussion

A DENV vaccine needs to elicit an immune response that is protective against the diverse strains that circulate for each serotype. Thus, it is important to characterize the antibody response elicited by a vaccine against heterologous strains. We previously studied the antibody response elicited by the DENV1 component of the NIH tetravalent vaccine candidate against DENV1 strain WP, the strain used in the vaccine. We determined that type-specific neutralizing antibodies in the polyclonal sera targeted epitopes containing residues E126 and E157 on DII and DI, respectively (261). While residues E126 and E157 are largely conserved among DENV1 strains, other residues important for recognition of epitopes comprised of these residues are not known. Notably, a DENV1 WP variant containing a mutation at residue K203 had reduced sensitivity to neutralization by several serum samples from DENV1 vaccine recipients, indicating that this residue is additionally important for recognition of DENV1 WP (**Figure 4.11**).

However, analysis of the ~1300 available DENV1 sequences revealed that <1% of DENV1 sequences contain a K at residue 203, while >99% contain an E. A K to E charge-change is likely to affect antibody recognition of epitopes in which residue 203 contributes to antibody binding. Therefore, if residue 203 is important for antibody recognition by antibodies elicited by the DENV1 WP vaccine, these antibodies may have reduced potency against 99% of heterologous DENV1 strains.

We investigated antibodies elicited against the DENV1 WP vaccine for potency against heterologous DENV1 strains 16007 and TVP2130. The greatly reduced potency of vaccine sera against TVP2130 compared to WP (**Figure 5.3**) may be due to amino acid variation within functionally important epitopes. While residues E126 and E157 are conserved, TVP2130 differs from WP at residue 203 and the neighboring residue, 202 (**Figure 5.1**). Of note, the substitutions at 202 and 203 are both charge changes. Residues 202 and 203 are located proximal to residue E126 (~10-15 Å away), and could potentially be part of E126-containing epitopes. TVP2130 differs at additional residues proximal to residue E126 (residues 225 and 88 are 9 and 19 Å away, respectively) and at a residue proximal to E157 (residue 155 is 3 Å away). Unlike the charge changes at 202 and 203, the substitutions at 88, 225, and 155 are chemically conservative; however, conservative differences still have the potential affect the potency of antibodies. Thus, the observation that the majority of the DENV1 sera had decreased potency against TVP2130 may be due to the amino acid variation at residues 88, 155, 202, 203, and/or 225. Characterization of TVP2130 variants encoding mutations at these positions would be needed to further explore hypothesis. Such a screen could potentially identify other

residues in the E126- and E157-containing epitopes that are important for antibody recognition.

Despite the decreased neutralization activity against WT TVP2130, some type-specific neutralization against this strain was observed: the majority of DENV1 serum samples neutralized WT TVP2130 >3-fold more potently than DENV2 (**Figure 5.6A**). Notably, the majority of the type-specific activity against TVP2130 was ablated by mutations E126K/E157K, similar to observations for strain WP (**Figure 5.6B**). Therefore, while type-specific antibodies in the DENV1 sera are less potent against TVP2130, the specificities that mediate type-specific neutralization appear to be conserved between WP and TVP2130.

In contrast to the reduced potency of the DENV1 sera against strain TVP2130, the majority of DENV1 sera neutralized strain 16007 with similar or greater potency than strain WP. That antibodies raised against strain WP were more potent against heterologous strain 16007 was surprising. Additionally, there is greater variation between the E proteins of strains 16007 and WP than between those of strains TVP2130 and WP (**Figure 5.1**). Like strain TVP2130, strain 16007 differs from WP at residues 202, 203, and 88 (proximal to E126). Strain 16007 additionally differs at residue 180 (20 Å from residue 157) and residue 204 (12 Å from E126). Despite the amino acid variation proximal to residue E126 and E157, only two serum samples had reduced potency against strain 16007 compared to strain WP (**Figure 5.3**). Furthermore, when mutations E126K/E157K were introduced into strain 16007, there was only a modest decrease in

the type-specific neutralization potency: the fold difference in titer relative to DENV2 was 18.4 for WT 16007 and 12.4 for 16007 E126K/E157K. The neutralization potency of the majority of serum samples was >3-fold greater against 16007 E126K/E157K than against DENV2. These results suggest that, while mutations E126K/E157K moderately impact neutralization of strain 16007, additional antibody specificities must be contributing to the type-specific neutralization of strain 16007.

The distinct antibody specificities that mediate neutralization of strains 16007 and WP may be due to the different structural dynamics of this strain. What these additional antibody specificities are is not known at this time. Determining the additional epitopes that are recognized on strain 16007 would require a screen of new 16007 variants, similar to the screen performed on strain WP. Additionally, the neutralization titers of nAbs that bind epitopes outside of DIII should be compared for WT 16007, 16007 R204K and other DENV1 strains.

That over half of the DENV1 serum samples displayed similar potencies against strains 16007 and WP (**Figure 5.3**) could suggest that the epitopes targeted by neutralizing antibodies in these samples are largely unaffected by differences in virion structural dynamics. If that were the case, then the same antibody specificities should be mediating neutralization of both strains. However, our data indicate that different antibody specificities mediate neutralization of strain 16007. An alternative explanation is that an increase in neutralization potency against strain 16007 due to structural dynamics may be offset by a decrease in neutralization potency caused by amino acid differences within

antibody footprints. As discussed above, strain 16007 varies from strain WP at several residues in DI and DII that are proximal to E126 and E157 (residues 88, 180, 202, 203). In this "sum of opposing forces" model, due to the polyclonal nature of sera, distinct antibody specificities could be mediating neutralization of strains 16007 and WP, yet result in similar potencies against the two strains. Characterization of additional 16007 variants could help elucidate what antibody specificities are involved in polyclonal antibody neutralization of strain 16007.

The three DENV1 strains studied within each displayed unique patterns of sensitivity to neutralization by DENV1 polyclonal sera obtained following monoclonal vaccination with a DENV1 candidate vaccine based on the WP strain. Whether this vaccine would be capable of mediating protection against distinct DENV1 strains is not known. Additionally, it would be interesting to compare antibody specificities elicited against different DENV1 strains. Do the distinct structural dynamics displayed by strain 16007 make it a better or worse immunogen? Would a DENV1 strain containing the more conserved glutamic acid at residue 203 offer broader neutralization potency against diverse DENV1 strains? The results of this study highlight the complexities involved in polyclonal antibody recognition of DENV.

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Chapter 6: Discussion[4]

The humoral response to viral infection has the potential to yield large numbers of B cells that produce antibodies with varied specificities, affinity for viral antigens, and functional properties. Considerable insight into how antibodies protect against infection, via direct neutralization of viral infection or antibody effector function, has come from studies of mAbs (57, 104, 130, 140, 141, 145, 172, 342-344). However, how antibodies function in concert as part of a polyclonal response is not well understood, nor is the breadth of the functionally significant components. For example, it is unclear why infection or vaccination may elicit antibodies that display neutralizing activity *in vitro* but contribute only modestly, if at all, to protection in the host. Two complementary approaches have been used successfully in an attempt to deconstruct the functional complexity of the polyclonal response. First, large panels of human mAbs have been created for many pathogens, allowing a detailed analysis *in vitro* and *in vivo* of the individual components of an antibody response. For flaviviruses, these approaches have yielded insights into how antibodies engage the many surfaces of the virion (50, 59, 60). Second, several studies, including the study outlined in this dissertation, have used novel molecular and biochemical approaches to identify epitopes recognized by nAbs within sera (54, 197, 261, 324-326, 345). Below, the findings from these two approaches are integrated and discussed in the context of the current understanding of the antibody response to

[4] Adapted in part from: **VanBlargan LA, Goo L, Pierson TC.** Deconstructing the Antiviral Neutralizing Antibody Response: Implications for Vaccine Development and Immunity. Manuscript submitted for publication.

flaviviruses and more broadly to other viruses, and how these findings can contribute to vaccine design.

6.1 Neutralizing Antibodies Are A Rare Component Of The Humoral Response Against Infection

Virus-specific human mAbs are identified by screening B cells for their ability to produce antibodies with desired functional properties. In many instances, candidate B cells, either MBCs or LLPCs, are screened for the ability to bind recombinant viral proteins (50, 346-348), virus-like particles (349, 350), or intact virions (57, 351) and then immortalized. Use of relatively high throughput neutralization assays has allowed functional screens to identify B cell clones that produce potent nAbs directly (352). The significant complexity of the antigenic surface of viruses suggests that the characteristics of the antigen used for screening, as well as the screening strategy will almost certainly impact the repertoire of mAbs identified (59).

In most instances, analyses of human anti-flavivirus mAbs suggest that antibodies with limited neutralizing activity comprise a large fraction of the virus-specific antibody response (50, 57, 59, 60, 353). For example, less than 5% of the antibodies isolated from flavivirus-infected or vaccinated humans displayed potent neutralizing activity (EC_{50} <0.5 mg/ml) (169, 301). A significant portion (~45-60%) of the total E protein-specific response appears to be directed against the highly conserved fusion loop in domain II (DII-FL) (169, 319, 343). Antibodies that target prM are also frequently isolated (50, 59, 60, 301). However, the majority of both DII-FL- and prM-reactive antibodies only

weakly inhibit infection (50, 168, 169, 171, 301, 343, 353). These antibodies bind epitopes that are either poorly accessible for antibody recognition (54) or are not present on virions with a stoichiometry sufficient to exceed the threshold requirements for neutralization (32, 54, 130). Similarly, although broad and potent nAbs against HIV-1 have been isolated from infected individuals (354), these nAbs represent a rare component of the overall humoral immune response. For example, a screen of 25 million peripheral blood mononuclear cells (PBMCs) from an individual whose serum displayed cross-reactivity against diverse HIV-1 strains identified only 3 somatically-related mAbs targeting the CD4 binding site that recapitulated the broad and potent neutralizing activity of the polyclonal serum (347).

Part of the challenge of eliciting effective nAbs against structurally complex viruses may be a requirement to engage a specific subset of the B cell repertoire. In support of this hypothesis, broadly neutralizing antibodies (bnAbs) against the HIV-1 CD4 binding site isolated from multiple donors appear to arise from a common, limited subset of germline genes that remarkably converge in sequence and structure during the affinity maturation process (348, 355-358). Antibodies elicited following acute DENV infection or influenza vaccination among unrelated individuals also display convergent sequence signatures (359, 360).

6.2 Neutralizing Antibodies Target A Limited Number Of Specificities

To complement studies of human mAb specificities, several groups have developed genetic and biochemical methods to investigate the contribution of particular epitopes to

the nAb response to flavivirus infection or vaccination. One approach is to compare the neutralizing activity of sera pre-incubated with soluble recombinant antigens representing various E protein domains to that of untreated sera. Such serum depletion studies have shown that DIII is not a major target of human nAbs following vaccination or natural infection with DENV (326), WNV (319), YFV (361), and TBEV (362), despite being the target of very potent murine neutralizing mAbs (47-49, 52, 56, 363, 364). These findings are supported by genetic approaches demonstrating that mutations in DIII epitopes do not result in a significant reduction in the neutralization potency of flavivirus immune sera from humans (319, 326). Most of the neutralizing activity of serum following YFV or DENV infection or vaccination is not affected by depletion by soluble E protein, suggesting that quaternary epitopes may be important targets for nAbs (326, 361), as discussed more below. In general, most studies characterizing anti-flavivirus nAb specificities in human polyclonal sera have largely ruled out the importance of particular epitopes, such as those in DIII and DII-FL (50, 60, 319, 326, 327).

The study outlined in this dissertation has identified residues on the E protein targeted by nAbs in polyclonal DENV-reactive sera. Epitopes targeted by nAbs elicited by a monovalent DENV1 vaccine candidate were investigated by creating and characterizing a comprehensive panel of DENV1 variants in which surface-accessible residues of the E protein were replaced by corresponding sequences of a DENV2 strain (261). This panel was screened to identify mutations that reduced sensitivity to neutralization by DENV1, but not DENV2, immune sera. This strategy identified two residues in DI and DII that when combined, ablated the DENV1 serotype-specific response to vaccination (**Figure**

4.10) (261). The idea that the anti-flavivirus immune response is focused on just a few specificities aligns with data from recent studies on HIV-1, in which most analyses of polyclonal sera with broadly neutralizing activity suggest that just one to two specificities can recapitulate the breadth and potency of the overall sera neutralizing activity (329, 365-367). Follow-up studies with multiple DENV serotypes will be needed to explore the fine specificity of type-specific nAbs.

Characterizing the kinetics and components of polyclonal sera over time may help define pathways to highly functional antibodies targeting critical epitopes. For example, there is increasing evidence that the continuous cycle of HIV-1 neutralization and escape from early strain-specific antibodies in sera often indirectly results in the exposure of epitopes that gradually lead to the induction of bnAbs during chronic infection (331, 368-371). It is possible that initial binding of antibodies to a particular epitope affects subsequent binding of other serum antibodies on the virion surface (149). This phenomenon may play a role in shaping the antibody repertoire that mediates potent neutralization. Additionally, the presence of antibodies bound to an antigen may affect subsequent antibody specificities that are elicited through epitope shielding. For example, distinct antibody specificities were elicited in mice following immunization with a TBEV E protein when the E proteins used for immunization were bound by antibody compared to unbound E proteins (362). It would be interesting to extend this finding to immunization with whole virus, as well as to secondary DENV infections, when presumably the virus is bound to some extent by cross-reactive antibodies from the primary infection.

6.3 Epitope Specificities Of Neutralizing Antibodies Inform Vaccine Design

Identifying the epitope specificities of protective antibodies to gain insight into strategies to elicit them via vaccination is the focus of rational vaccine design efforts for many viruses (372-375). Because of the complex and dense arrangement of flavivirus E proteins on the virion surface, not all epitopes are equally accessible to antibodies (296). Antibodies that bind poorly accessible or cryptic epitopes are characterized by greater occupancy requirements for neutralization and are capable of enhancing the infection of FcR-expressing cells at high concentrations (50, 60, 130, 376). This enhancement of infection has been linked mechanistically to more severe clinical outcomes of DENV infection (12, 84). Thus, a flavivirus vaccine that elicits antibodies targeting accessible epitopes may be more optimal for providing protection. However, as discussed below, depending on the biology and pathogenesis of the virus, accessible epitopes may not always be ideal targets for the most protective antibodies. The characterization of large panels of potently neutralizing mAbs has greatly improved our understanding of the molecular basis of antibody recognition and neutralization and has the potential to inform vaccine design.

6.3.1 Accessible epitopes: easy targets.

Neutralization of flaviviruses is governed by a requirement for "multiple hits" whereby antibodies that target highly accessible epitopes can achieve neutralization at a lower occupancy compared to those targeting poorly exposed epitopes (130). In support of this, many of the most potent mAbs characterized to date bind highly accessible epitopes on DIII (47, 49, 377). For example, the murine WNV-specific mAb E16 binds a

discontinuous epitope in the lateral ridge of DIII (DIII-LR), neutralizes at picomolar concentrations *in vitro* (364), and prevents WNV-induced mortality in animals when given as a single dose after infection (364, 378). Cryo-electron microscopy studies demonstrate that E16 can bind its epitope on 120 out of 180 E proteins on the mature virion (144), allowing antibody binding to exceed the stoichiometric threshold for neutralization at low occupancy (130) (**Figure 6.1A**).

In the study outlined in this dissertation, residues selected for mutagenesis were surface exposed on the structure of the mature virus. Thus, this strategy biased the screen to identifying residues in epitopes that are (at least partly) accessible. That this strategy was successful in identifying the principal targets of the type-specific neutralizing antibody response to DENV1 vaccination suggests that functionally significant antibodies elicited by the vaccine likely target epitopes with exposed residues.

6.3.2 Cryptic epitopes: dynamic targets.

In contrast to highly potent antibodies that target accessible epitopes, antibodies that bind hidden epitopes often mediate neutralization only at very high concentrations. However, viral factors that modulate epitope accessibility, such as the efficiency of virion maturation and viral "breathing", contribute significantly to the ability of antibodies to recognize cryptic epitopes (32, 34, 138, 172). For example, DENV1 mAb E111 recognizes an epitope on DIII predicted to be inaccessible on the mature virion (**Figure 6.1B**), and is weakly neutralizing against the majority of DENV1 strains tested under standard neutralization assay conditions (172). Despite this, E111 can achieve potent

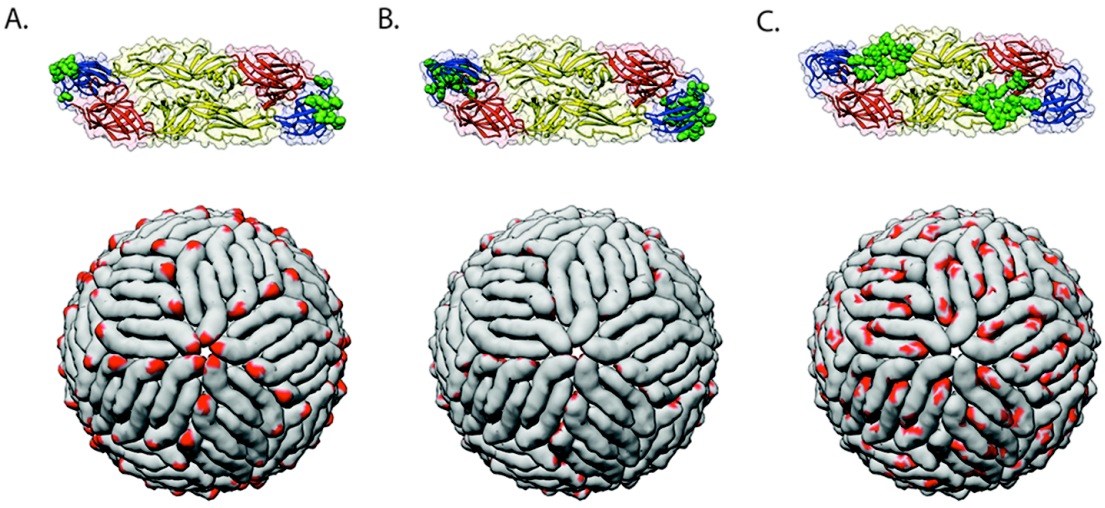

Figure 6.1 Examples of epitopes targeted by anti-flavivirus monoclonal antibodies. Epitopes of selected mAbs are shown in green on the E protein dimer (PDB: 1OAN) (top panel) or in red on the mature virion (PDB: 4CCT) (bottom panel). (A) Monoclonal antibody E16 targets the DIII lateral ridge, a highly accessible epitope on the virion surface (144). (**B**) Monoclonal antibody E111 recognizes a cryptic epitope on DIII that is poorly accessible on the mature virion (172). This mAb may rely on virus "breathing" for epitope accessibility. (**C**) Monoclonal antibody EDE2-B7, targets a quaternary epitope that spans adjacent E protein monomers at the dimer interface (191).

neutralization of DENV1 following increases in the time and temperature at which virus-antibody immune complexes are incubated, which allows for virus "breathing" to expose the E111 epitope for antibody binding (172). Two additional DENV-specific mAbs that similarly recognize cryptic epitopes are the murine mAbs 4E11 (379) and 1A1D-2 (51). Both mAbs target an epitope centered on the A-strand of DIII-LR that is not predicted to be accessible for antibody recognition on mature virions (173), as the fusion loop is inserted into the hydrophobic pocket near the DIII A-strand on the partner E protein in the dimer, thus theoretically blocking the binding site of 4E11 and 1A1D-2 (334). Presumably, these mAbs bind and stabilize the A-strand epitope as it becomes transiently available on the "breathing" virion. In support of this, 1A1D-2 has been shown to bind to the DENV virion at 37°C, but not at room temperature, suggesting that temperature-dependent increases in the mobility of E proteins on the virion surface promote accessibility of the A-strand epitope (173).

Virion maturation state also plays a role in modulating the exposure of cryptic epitopes. For example, the WNV-specific murine mAb E53 binds an epitope that includes residues in the DII-FL and adjacent BC loop (171). This mAb has almost no neutralizing activity against fully mature WNV virions, but displays increased neutralization potency against virions that retain uncleaved prM on the surface (32). Cryo-electron microscopy studies revealed that the E53 Fab binds preferentially to spikes on immature flavivirus virions, providing a structural basis for the maturation-state dependence of E53 neutralizing activity (170).

Although the presence of a stoichiometric threshold of neutralization suggests that highly accessible epitopes are ideal targets for potent nAbs, for viruses with extensive genetic and antigenic heterogeneity such as HIV and influenza, epitopes that are highly exposed are often highly variable. Potent nAbs can develop against hypervariable loops that shield more conserved regions in the HIV envelope (166), but these antibodies tend to be highly strain-specific, and rapidly select for escape variants (reviewed in (380)). An enormous challenge in developing an effective vaccine against rapidly evolving viruses is the requirement to elicit potent antibodies against conserved sites that are often occluded and are thus poorly immunogenic (reviewed in (381)). Nonetheless, a number of conserved vulnerable sites on the HIV envelope that are recognized by broad and potent nAbs have been identified. These include the CD4 binding site (347, 355, 382), glycan-dependent epitopes in V1/V2 (352) and V3 (383) of gp120, the membrane proximal external region (MPER) (384) of gp41, and most recently, epitopes spanning both gp120 and gp41 subunits (385-388).

In contrast to the apparent accessibility of DIII-LR epitopes that elicit strongly neutralizing, virus-specific antibodies against flaviviruses, epitopes that induce broad and potent nAbs against HIV are often buried under a dense shield of glycans or are deeply recessed on the native trimer (389, 390). However, the remarkable potency of some bnAbs against HIV suggests that such epitopes are exposed frequently enough to allow antibody binding to exceed the neutralization threshold. Alternatively, such potency may reflect the unusual characteristics displayed by most HIV bnAbs identified to date, such as extensive somatic hypermutation, long CDR-H3 loops, and the ability to recognize

glycans (reviewed in (354)). For example, the broadly neutralizing mAb VRC01 encodes 41 V_H gene alterations and 25 Vκ gene alterations from the germline sequence (390). A separate study found that only one percent of V_H genes from IgG memory and GC B cells had greater than 40 mutations (94). Further identification and characterization of additional HIV antibodies and epitopes will help clarify whether these unusual characteristics are required to achieve broad and potent neutralization (179, 391).

6.3.3 Quaternary epitopes: complex targets.

Many nAbs target complex, conformational, surface-accessible epitopes that are only optimally exposed on an intact virion, but not on soluble envelope proteins. For example, a recent analysis of 145 human mAbs isolated from seven DENV infected patients found that over 40% of the mAbs were only reactive against whole virions, and not against recombinant E protein (191, 392). Most of these virion-specific antibodies target an epitope that overlaps the DII-FL epitope and bridges the E dimer interface (E-dimer dependent epitope; EDE) (**Figure 6.1C**) (191, 392). Importantly, these mAbs display very potent neutralization across all four DENV serotypes (191, 392). The specificity of the EDE mAbs differs from that of most of the other potently neutralizing, anti-flavivirus mAbs targeting quaternary epitopes across adjacent E protein dimers. These latter antibodies often recognize epitopes that overlap the DI/DII hinge, and include the human anti-WNV mAb CR4354 (145), and anti-DENV mAbs 5J7, 1F4 (57), and HM14c10 (58). The prevalence of nAbs targeting complex epitopes is not limited to those against flaviviruses, as many of the most potent HIV-1 bnAbs have been found to recognize similarly presented epitopes (352, 385-388). It is possible that the neutralization potency

of antibodies targeting these complex epitopes is due to their ability to bind to sites that are only exposed on the most functional configuration of the viral envelope.

The mutagenesis strategy outlined in Chapter 4 identified individual residues, not entire epitopes, that were targeted by the polyclonal antibody response. Because other residues contributing to epitopes containing residues 126 and 157 were not defined, it is not known whether residues 126 and 157 are part of quaternary epitopes. Notably, however, many of the human mAbs isolated to date that bind nearby epitopes in DI and/or DII recognize quaternary epitopes (57, 58, 145, 342). A type-specific, potently neutralizing human mAb, 1F4, was recently characterized that recognizes an epitope on DI of DENV1, including residue E157 identified in our study (393). While the epitope of 1F4 is entirely contained within one E monomer, 1F4 binds whole virions but not recombinant E protein, suggesting its epitope is quaternary structure-dependent (57, 393).

The discovery of potent nAbs that recognize quaternary epitopes exposed only on the virion surface has important implications for the identification and analysis of functional components of the polyclonal antibody response against viruses. In addition to the apparent rarity of B cells that contribute to the neutralizing component of the antibody response, one possible explanation for the discrepancy between dominant binding versus neutralizing epitope specificities is that many antiviral nAbs target quaternary epitopes that are only optimally exposed on the virion surface, and are thus not faithfully represented on soluble recombinant proteins that are often used in binding studies.

6.4 Future directions

In this dissertation, the human, type-specific, neutralizing antibody response to a live attenuated, DENV1 monovalent vaccine was investigated. The mutagenesis strategy developed within to identify functionally important targets of the antibody response could be applied to future studies to investigate and compare antibody responses to different types of DENV1 vaccination and infection events. It would be of interest to compare the antibody specificities elicited by the NIH DENV1 vaccine studied within to infections with heterologous DENV1 strains. It would also be of interest to compare antibody responses to different types of immunogens, such as viruses that differ with respect to maturation state or conformational dynamics (such as DENV1 strains WP and 16007). Additionally, antibody responses to different types of vaccines could be compared, such as live-attenuated vaccines versus inactivated virus, recombinant E protein, or DNA vaccines.

Similar systems could be easily developed to study the other DENV serotypes and used to identify whether neutralizing antibodies against heterologous serotypes targets similar regions of the E protein. These systems could also be used to investigate how the antibody response is skewed following tetravalent vaccination or following sequential exposures such as after secondary infections or sequential heterotypic vaccination (394).

In addition to the neutralizing abilities of DENV Abs, effector functions such as ADCC may be involved in DENV protection or pathogenesis (302-304). Antibody specificities capable of mediating ADCC of DENV-infected cells are not known. While our system

for studying the neutralizing potency of antibodies is not able to measure antibody effector functions, current ADCC assays may be modified using aspects of our system for investigating ADCC-mediating Ab specificities.

6.5 Concluding Remarks

Much of our mechanistic understanding of virus/antibody/host interactions has been gleaned from the study of mAbs. However, a polyclonal antibody response against a particular pathogen may contain a diverse array of antibodies that can differ drastically with regards to epitope specificity, neutralizing potency, effector function, and breadth of recognition (narrow type-specificity vs. broad cross-reactivity). Within this diverse repertoire of a polyclonal response, the fraction of antibodies that contribute substantially to protection may be small.

As different viruses employ multiple distinct antibody evasion strategies, the epitope diversity required for optimal protection may vary among viruses. Because of the potential for non- or weakly neutralizing antibodies to contribute to pathogenesis of DENV infection (81), eliciting antibodies against accessible epitopes (including quaternary determinants) that allow high occupancy binding at low concentrations may be ideal (130). In contrast, for viruses that exhibit more extensive antigenic variability, such as HIV-1 and influenza, an effective vaccine may need to elicit potent nAbs against hidden epitopes that are conserved across multiple variants. The ability of these viruses to rapidly escape from immune pressure may suggest a role for nAbs with multiple specificities in affording protection. Indeed, many studies have shown that no single

antibody specificity is likely to be effective in providing protection against diverse HIV-1 variants, but combining antibodies that target distinct neutralization epitopes increases overall neutralization coverage (383, 395-400). Currently, it is unclear whether eliciting broad and potent nAbs against multiple epitopes via vaccination is feasible, although there is evidence that such nAbs can develop within a single individual during HIV-1 natural infection (329, 401, 402).

Identifying the specificities of nAbs elicited following vaccination or natural infection may guide immunogen design to more effectively engage the subset of naïve B-cell receptors that lead to broadly protective functional responses. This information is especially relevant given recent advances in deep sequencing technology coupled with proteomic analysis of antigen-specific IgG that allow the isolation and characterization of functionally relevant antibodies from polyclonal sera (403, 404). Thus, it has become possible to enrich for epitope-specific nAbs and characterize their ontogeny to inform vaccine design (348, 355)). Finally, there is growing evidence that functionally relevant epitopes grafted onto molecular scaffolds can serve as promising immunogens (374, 405).

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